STUDIES ON THIAMINE TRANSPORT IN *ESCHERICHIA COLI* CROOKES

By

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STUDIES ON THIAMINE TRANSPORT IN ESCHERICHIA COLI CROOKES

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Dedicated to my husband Kermit, whose challenges have stimulated my initiative and whose dedication to science has inspired me.

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CHAPTER I

INTRODUCTION

The plasma membrane is a complex entity with a multiplicity of functions, including selective permeability to molecules and ions in the extracellular environment. This selectivity has as its basis the structure and organization of membrane components and the presence of specific transport components for a sizeable number of nutrient molecules and ions necessary for cellular function.

Several types of transport have been defined and studied in a wide variety of organisms. Pinocytosis and phagocytosis have not been found in microorganisms (1); therefore these modes of transport will be omitted from the following discussion. Coupled transport, i.e., influx or efflux of an ion or compound via coupling with Na^+ or K^+ flow mediated by the Na^+, K^+-ATPase, occurs widely in higher organisms but, though found in a very few simpler organisms (2,3), is not prevalent in microorganisms.

The primary modes of transport in microbes include simple diffusion, facilitated diffusion, active transport and group translocation. Simple or passive diffusion involves diffusion of solute across a membrane without mediation by membrane components. The process does not require metabolic energy but is dependent upon an electrochemical gradient and is non-stereospecific. The rate of penetration is a function of such parameters as membrane thickness and diffusion coefficient as well as

concentration gradient. Simple diffusion of most important nutrients is not regarded as a significant physiological process. Facilitated diffusion is similar to passive diffusion in that solute moves down a concentration gradient; there is no energy requirement other than that necessary for maintenance of membrane integrity. However the process is catalyzed by a membrane component, therefore the rate is more rapid than predicted for passive diffusion. The catalytic component is stereospecific, and the process exhibits saturation kinetics. Active transport is also mediated by a carrier; however, transport of solute occurs against a concentration gradient, consequently requiring energy expenditure. Group translocation occurs when transported solute undergoes concomitant chemical reaction, such as phosphorylation. This process also requires metabolic energy.

Although concentration of specific substrates against a gradient has long been recognized, the existence of carriers was not demonstrated until the mid-1950's. In 1955 Cohen and Rickenberg (4) showed that in Escherichia coli β-galactoside transport and hydrolysis are separate functions, although the activities are jointly induced by β-galactosides in the medium. Cohen and Monod (5) postulated the existence of a protein distinct from the β-galactoside hydrolyzing enzymes which was responsible for increased transport on induction. The controversial name permease was bestowed upon this protein and suggested for all other proteins or protein systems which perform such a carrier function (6).

The fact that bacteria accumulate substrates against a concentration gradient provided evidence for such a permease. Bacterial cells also show crypticity, i.e., inability to grow on specific substrates despite the presence of metabolic enzymes for utlization of those substrates.

That the cells grow on other stereoisomers of the substrates indicates that these permeases exhibit stereospecificity. Cohen and Monod (5), using inhibitors which convert normal cells into cells demonstrating phenotypic crypticity, found a great reduction in permease activity and a corresponding reduction in substrate hydrolysis, whereas the in vitro β-galactosidase activity remained constant. Thus they demonstrated that crypticity is due to selective permeation rather than selective utilization of substrate by metabolic enzymes. They also showed that accumulation of substrate is independent of utilization and that accumulation is highly stereospecific. Since the transported substrate was osmotically active within the cell, they concluded that accumulation is the result of a catalytic rather than a stoichiometric permease (5).

These observations stimulated investigations on many other transport systems. Studies on amino acid transport systems have shown that almost all are active transport systems having a high affinity for substrate and showing a great degree of specificity. Energy dependence for many of these systems has been demonstrated by the uncoupling of the facilitated diffusion portion of the transport activity from the active process by low temperature or inhibitors of energy production. Many of the K_m values are in the micromolar concentration range, although there are a few less specific low-affinity systems, such as histidine transport by the aromatic permease in Salmonella typhimurium (7).

Transport systems for many Krebs cycle intermediates (8,9,10), glucuronides (11) and several other carbohydrates were described during this time. Reviews by Cohen and Monod (5), Kepes and Cohen (12) and Hendler (13) provide detailed explanations of these systems.

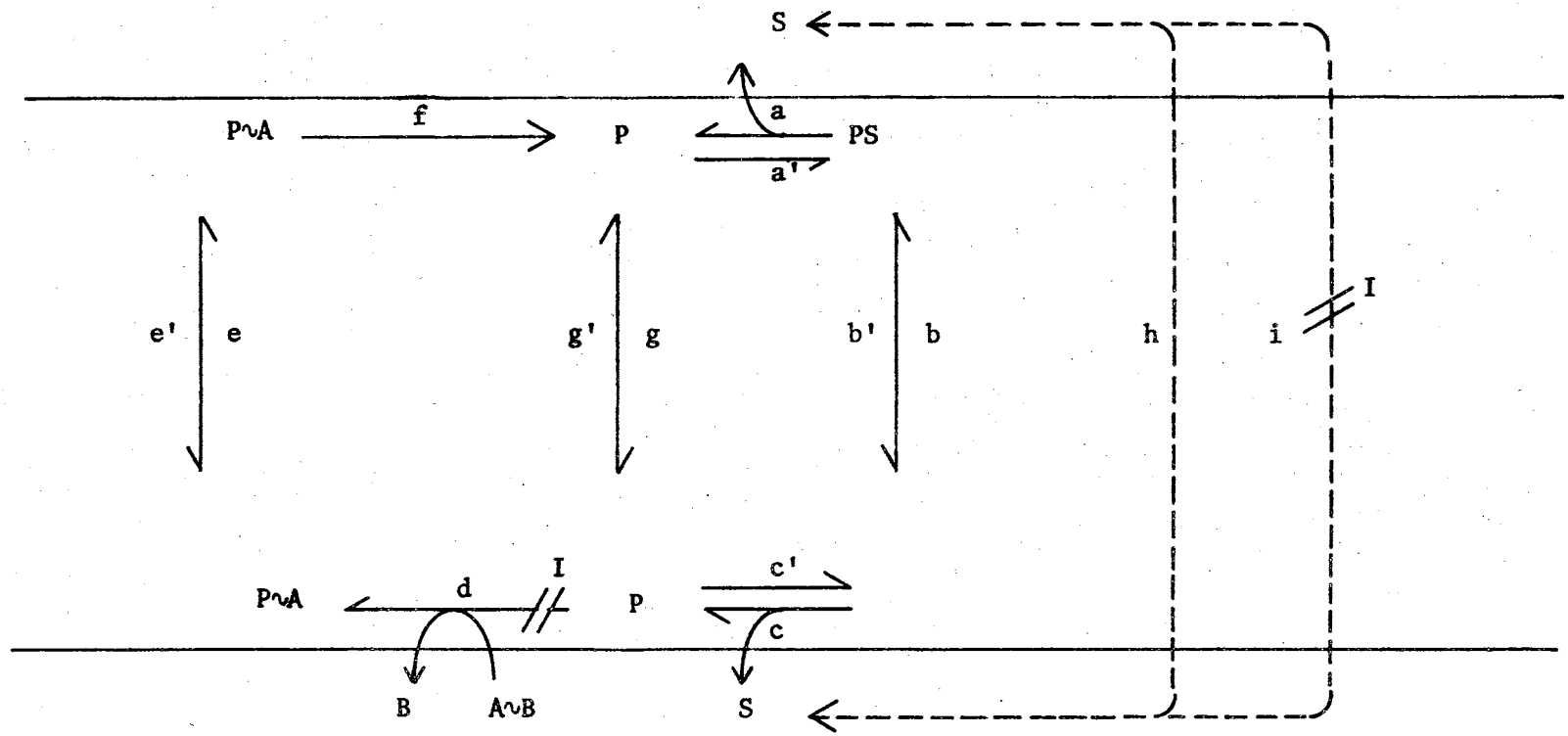
It was the massive attack on the β-galactoside system, including

influx and efflux studies, which afforded enough information for the postulation of the first detailed transport model by Kepes in 1960 (14). This model, as modified by Koch in 1964 (15) to accomodate energy inhibitor effects on influx or efflux, proposed the existence of a nonspecific, constitutive mobile carrier which could exist either in an energized state or in a low-energy carrier-substrate complex. A specific permease catalyzes the formation of the carrier-substrate complex, with energy required for the dissociation of this complex (15). This interpretation of the site of energy requirement differs from that proposed by Kepes (14), who postulated requirement for energy in the permease-mediated carrier-substrate formation. Winkler and Wilson (16) substantiated Koch's site of energy requirement hypothesis, finding that energy sources decreased the affinity of carrier for substrate in the carrier-substrate complex on the interior of the cell. However, their studies suggested that the carrier and permease were the same and that the permease was involved in both active transport and facilitated diffusion. A number of refinements of this model have been made (17,18) such that the rather complicated scheme shown in Figure 1 represents a model consistent with the information amassed to date on the β-galactoside transport system (18). This author has added membrane boundaries, letters for reverse reactions and site of inhibitor action to facilitate explanation of the model.

The model is based on the experimental facts described above and the following three postulates. The first is that the energy coupling step involves a covalent reaction between energy donor and permease protein resulting in P∿A, the energized form of the permease. The second is that energy coupling is comprised of two steps, the covalent reaction and an

Figure 1. The Kepes Galactoside Permease Model

Membrane-located permease protein P can assume two orientations, active site outward and active site inward. The transition between the two is designated g or g'. Outward-oriented P can combine with substrate S to form PS complex, with dissociation of the complex proceeding by a'. PS (outward) can undergo a reversible orientation change to PS (inward). PS (inward) can dissociate reversibly to release S into the cytoplasm. P (inward) can then undergo an energy-coupled (thermodynamically irreversible) reaction, forming an energized P∿A complex. P∿A can then undergo an orientation change via reaction e and an exergonic, energy-dissipating reaction f, regenerating P (outward). Galactosides can also be transported by non-permease systems h and i (or possibly more), one (or more) of which can be inhibited by energy poisons. b, g, e, h and i represent some type of translocation; a, c, d and f are biochemical reactions.


S
P∿A
f
P
a
PS
a'
e'
e
g'
g
b'
b
h
i
I
P∿A
d
I
P
c'
c
B
A∿B
S

energy dissipation step which involves breakage of the covalent bond. The third is that the permease can exist in a number of forms, such as free P, complexed with substrate PS or energized P∿A, all of which are capable of inward or outward orientation of the binding site. All configurations must be transformable such that the total rate of transformation of inward-oriented forms into outward-oriented ones are equal to the total rate of transformation in the opposite direction.

In 1964 Kundig, et al. (19) discovered in E. coli a transport system energized by phosphoenolpyruvate which was involved in the accumulation of a number of sugars. In Gram-negative organisms this phosphoenolpyruvate (PEP) phosphotransferase is comprised of three components: a soluble, heat stable, low molecular weight protein HPr; Enzyme I, which is soluble, constitutive and non-specific; and Enzyme II, an inducible specific membrane-bound component (19,20,21). In Gram-positive organisms a fourth component, Factor III (21), is required for phosphorylation of the sugar on which the cells are grown. It is an inducible cytoplasmic protein which reduces the affinity of Enzyme II for the sugar for which it is specific.

The sequence of reactions which the PEP phosphotransferase system catalyzes is:

$$\text{PEP} + \text{HPr} \xrightleftharpoons[\text{Mg}^{2+}]{\text{Enzyme I}} \text{P-HPr} + \text{pyruvate}$$

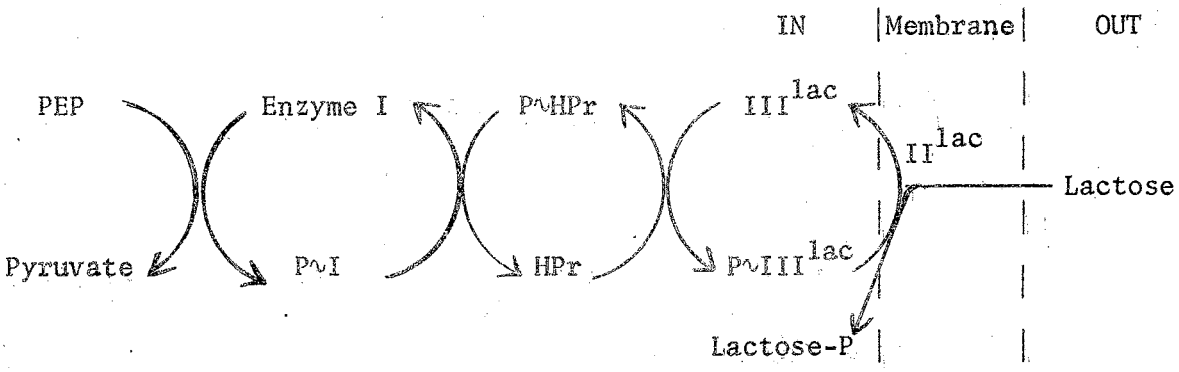
$$\text{P-HPr} + \text{hexose} \xrightarrow[\text{Mg}^{2+}\ (\text{III})]{\text{EnzymeII}} \text{hexose-6-P} + \text{HPr}$$

$$\text{PEP} + \text{hexose} \xrightarrow[\text{Mg}^{2+}\ (\text{III})]{\text{Enzymes I and II}} \text{hexose-6-P} + \text{pyruvate} \quad .$$

Phosphate is transferred to a histidine residue of HPr to form the intermediate P-HPr in a Mg^{2+}-requiring reaction mediated by -SH-containing Enzyme I. The phosphate carrier then transfers phosphate to the sugar in a reaction catalyzed by Enzyme II (20,21).

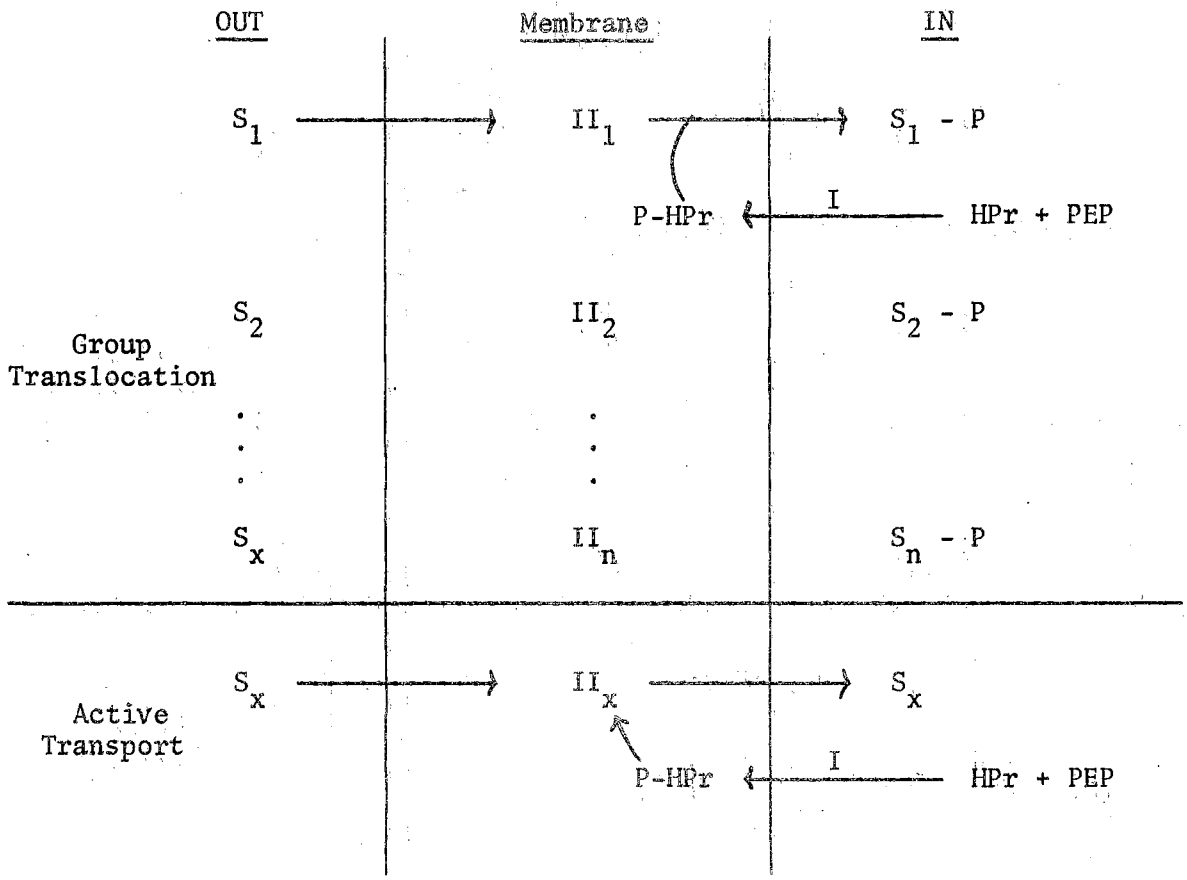
The PEP phosphotransferase system has been demonstrated in a number of organisms. It participates in the transport of a large number of sugars in Staphylococcus aureus, including α-methyl glucoside, glucose, mannose, fructose, glycerol, sorbitol, mannitol, N-acetylglucosamine, maltose, melibiose, galactose and lactose (22). However, in E. coli relatively few sugars (i.e., glucose, mannose, fructose and mannitol (23)) are transported by this system.

The lactose transport system in S. aureus, which catalyzes the transport of both galactose and lactose, has been purified and characterized (24,25,26). The following schematic diagram (24) represents the reactions involved in phosphate transfer from PEP to lactose in this organism.



The diagram in Figure 2 shows mechanisms by which different types of transport may occur (22). This model stresses the fact that separate enzymes II are required for different substrates. According to Simoni, et al. (26), phosphorylation of substrate is not a requisite of this

Figure 2. The Roseman Phosphoenolpyruvate Phosphotransferase Transport Model


OUT
Membrane
IN
S_1
II_1
S_1 - P
P-HPr
I
HPr + PEP
S_2
II_2
S_2 - P
Group Translocation
S_x
II_n
S_n - P
S_x
II_x
S_x
Active Transport
P-HPr
I
HPr + PEP

model; transport of unchanged substrate may be mediated by a phosphorylated enzyme II.

Kepes (24) has pointed out the lack of correlation between sugar transport rates and intracellular levels of ATP. This observation suggests that the control mechanism for PEP-phosphotransferase-mediated transport lies in the glycolytic pathway. Anaerobic glycolysis of cells in a phosphate-sufficient medium is controlled by availability of ADP. In cells which are rapidly respiring but have limited ATP demand most of the available adenosine nucleotide exists as ATP. Thus, the lack of ADP halts glycolysis, and despite maximal levels of ATP, PEP is not synthesized and transport stops (27). These considerations explain the behavior of inhibitors on PEP-phosphotransferase-mediated transport. The transport rate of methyl-α-glucoside is greatly stimulated rather than inhibited in the presence of concentrations of azide and dinitrophenol, which should uncouple oxidative phosphorylation (28). The explanation for this observation is that uncoupling of oxidative phosphorylation increases the rate of formation of PEP (29). Under conditions where uncouplers stimulate transport the anaerobic glycolysis inhibitor iodoacetate markedly inhibits the rate (28).

Roseman (22) cites a teleological explanation for the utilization of PEP rather than ATP for energization of a transport system. First, PEP participates in fewer reactions than does ATP, so that the internal pool of PEP is more easily regulated. Second, the rate of PEP synthesis depends upon the rate of anaerobic glycolysis, which is relatively independent of variations in the degree of aerobiosis; this would not be true for ATP. Third, energy may be conserved in the phosphorylation of substrate, which will afford two ATP's from anaerobic glycolysis rather than

the net one ATP obtained if phosphorylation-dephosphorylation occurred.

At the time the original PEP-phosphotransferase model was postulated proof for the existence of permease systems had been demonstrated, and the next logical step was the isolation and characterization of a transport component. In 1965 Fox and Kennedy (30) made the first major breakthrough in purifying and characterizing such a component. Kepes (14) had shown that the β-galactoside transport system was inhibited by sulfhydryl reagents. Treatment of energy-poisoned cells with N-ethylmaleimide (NEM) (30) produced a striking inhibition of rate of hydrolysis of the non-metabolizable β-galactoside analog o-nitrophenyl-β-galactoside without inhibition of β-galactosidase or thiogalactoside transacetylase, the hydrolases which are the other two products of the lac operon. Fox and Kennedy (30) postulated that the component inhibited by NEM was the carrier protein. Since the equivalence of the permease and the NEM-inhibited component had not been established, they referred to the component as the M protein.

If the M protein were indeed the NEM-inhibitable component of the transport system, these authors hypothesized that it might be possible to protect this component from inactivation by addition of the substrate thiodigalactoside, for which the transport system has a high affinity. Such protection was found and provided the basis for an elegant double-labeling experiment which demonstrated that the M protein was indeed the component inhibited by sulfhydryl reagents.

Since the β-galactoside transport system is inducible, induced cells should contain more of the transport component than uninduced cells. A double labeling experiment was performed in which thiodigalactoside-protected non-induced and induced cells were labeled with ^{3}H-NEM and

^{14}C-NEM, respectively. The two cultures were mixed, sonicated and centrifuged. The supernatant fraction containing the hydrolases showed no enrichment of ^{14}C, as would be expected if either of these were the inducible NEM-inhibitable component involved in the increased transport found in induced cells. However, the particulate fraction did show enrichment of ^{14}C label. The membrane-bound M protein was extracted with nonionic detergent. Later studies with spheroplasts (31) substantiated the observation that the M protein was not a periplasmic protein, but rather was a firmly bound membrane component.

Almost simultaneously Pardee and Prestidge (32), studying sulfate transport in S. typhimurium, detected a sulfate binding protein in cell-free extracts obtained by sonication of whole cells. However, since most binding proteins comprise such a small fraction of total cellular protein, isolation from whole cell sonicates is difficult. Neu and Heppel (33) devised an osmotic shock treatment of cells involving treatment with Tris-EDTA in hypertonic sucrose and subsequent cold osmotic shock in distilled water or dilute $MgCl_2$. A relatively small amount of protein is released in this manner, and a number of enzymes and binding proteins have been isolated from cells treated in this manner.

A growing body of work is aimed at demonstrating involvement of these shockable binding proteins in their respective transport processes. Varying degrees of success have been obtained, some of the more convincing being reconstitution of transport ability lost on osmotic shocking by addition of isolated binding protein. Relatively few of these reconstitution attempts have been successful (34,35,36,37). Behavior of binding proteins with respect to repression, inhibition and affinity for substrate indicate that they may be involved in transport. Studies in

which mutants lacking a specific transport function also lack the corresponding binding protein implicates the binding protein in transport. These types of evidence are somewhat circumstantial, but if indeed binding proteins are involved, it is rather generally concluded that they are not the sole mediators of transport.

Studies on isolated binding proteins have afforded information which suggests that other components also play a role in the transport process. Binding proteins are easily removed from the cell and are soluble in aqueous medium. On the other hand proteins such as the M protein (30) which are tightly bound to the membrane have also been implicated in transport. Binding of substrate to binding proteins is independent of metabolic energy and reversible and occurs readily at low temperatures (38,39). Addition of high-energy compounds affects neither binding kinetics nor reversibility. On the other hand in vivo active transport is both energy- and temperature-dependent. Active transport does not occur at 4° or in the presence of energy inhibitors (8,35,40,41). Moreover, Anraku (34) has shown that specific proteins which have no binding activity are involved in reconstitution of transport. Evidence for other transport components is also provided by the isolation of transport-negative mutants which retain full complement of binding protein (42).

Kerwar, et al. (43), in their study of galactose transport in isolated membranes of E. coli, have demonstrated that the galactose binding protein is not required for transport. These membranes did not contain galactose binding protein detectable by anti-serum but did transport galactose. However, this particular situation is complicated by the presence of as many as four transport systems for galactose (44), and the binding protein may be required for no more than one of them.

No soluble transport component has been found for the proline transport system unless cells are grown in tryptone medium (45); all activity is tightly membrane-bound and cannot be removed by such extreme treatment as sonication (46). However, Gordon, et al. (47), using membrane-disrupting nonionic detergents, have solubilized and partially purified a fraction from membrane vesicles which binds proline. The proline-binding activity is inhibited by sulfhydryl reagents, and PCMB inhibition is reversed by dithiothreitol. These properties are analogous to those of the proline transport system observed in intact vesicles. Electron transport and general metabolic inhibitors do not affect proline binding by this fraction. The fraction also binds other amino acids with a low K_d, including lysine, serine, tyrosine and glycine.

There is a distinct difference between the membrane components solubilized by chaotropic agents, such as the amino acid-binding fraction just described and the M protein purified by Fox, et al. (30), and binding proteins released by osmotic shock. Binding proteins are localized in the periplasmic space and therefore are water-soluble; most contain no cysteine and are insensitive to sulfhydryl reagents. In contrast the membrane-bound components are soluble only in detergent and are sulfhydryl reagent-sensitive.

Consequently the next logical step in approaching the elucidation of the transport process is a study of the membrane-bound components in situ and their possible interactions with binding proteins and with membrane components which are indirectly involved with transport, e.g., the respiratory chain.

Kaback and co-workers reported that subcellular preparations (48) and cell-free preparations from E. coli catalyzed the uptake of glycine

(48,49) and of proline (50,51). These membrane studies established a model system which simplifies the study of transport. Methods for preparation of the bacterial membrane vesicles and their characterization with respect to homogeneity, composition and physical properties have been reviewed in detail (52,53) and will be discussed more thoroughly where appropriate in the body of this thesis.

The PEP phosphotransferase catalyzes the vectorial phosphorylation of glucose and related monosaccharides in E. coli, S. typhimurium and Bacillus subtilis membrane vesicles (52,53,54,55,56). Free sugar is accumulated in the vesicles as sugar-P without mediation of an intramembranal free sugar pool. Little glucose-P is taken up with or without PEP in the medium. As mentioned previously, this system is involved in transport of only a few other sugars in E. coli, nor is it involved in most inducible sugar or amino acid transport systems.

Studies on proline uptake in E. coli membranes have supplied information on energetics which provides the basis for a totally different mode of transport from the PEP-phosphotransferase system (57). D-Lactate addition to vesicle suspensions stimulates proline uptake 20- to 30-fold. Only succinate, L-lactate, D,L-α-hydroxybutyrate and NADH, of all the metabolic intermediates and cofactors tested, could replace D-lactate, and all are much less effective than D-lactate. D-[^{14}C]Lactate, L-[^{14}C]lactate and D,L-[^{14}C]lactate are converted stoichiometrically to pyruvate and [^{14}C] succinate to fumarate (55,56,57). The [^{14}C] pyruvate is not further metabolized by the vesicles, and neither pyruvate nor fumarate affects proline transport.

These results indicate that proline transport involves electron transport, and that a membrane-bound lactate dehydrogenase specific for

D-lactate is tightly coupled to proline transport.

The rate and extent of conversion of lactate to pyruvate in the vesicles was much greater than could be accounted for by proline transport alone. For this reason the effect of D-lactate on transport of other amino acids was investigated. The results (57) showed that conversion of D-lactate to pyruvate markedly stimulates both initial rates and steady-state levels of uptake of proline, glutamic acid, aspartic acid, tryptophan, serine, glycine, alanine, lysine, phenylalanine, tyrosine, cysteine, leucine, isoleucine, valine, and histidine. All of the radioactivity taken up was recovered as the unchanged amino acid in all cases (57). The observations noted above on replacement of D-lactate by other energy sources have been extended to all of these amino acid transport systems (58), although the relative effects on a particular transport system vary (56).

Since attempts to implicate the PEP-phosphotransferase system in the transport of galactosides by isolated membrane vesicles were uniformly negative, the effect of D-lactate on β-galactoside uptake by vesicles was investigated (59). In membrane vesicles prepared from cells containing a functional y gene the conversion of D-lactate to pyruvate markedly stimulated the initial rate of β-galactoside uptake and promoted 100-fold accumulation. Galactoside-P was not detected, and virtually all radioactivity was recovered as unchanged substrate. Only D-lactate, D,L-α-hydroxybutyrate, succinate and L-lactate stimulate lactose transport above endogenous levels, and NADH has no effect on this system. Other sugar transport systems coupled to D-lactate dehydrogenase are galactose (43), arabinose, glucuronic acid, gluconic acid and glucose-6-phosphate (1).

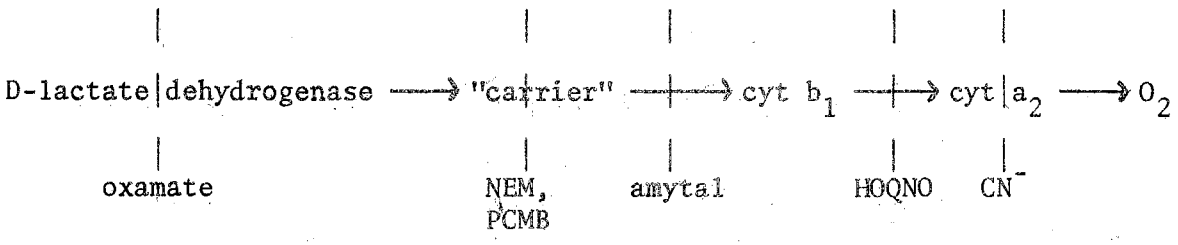
Barnes and Kaback (59) demonstrated that in membrane vesicles there is no relationship between oxidation of D-lactate, succinate, L-lactate and NADH and the ability of these electron donors to stimulate transport. In vesicles prepared from succinate-grown cells succinate is oxidized much more rapidly than D-lactate and NADH is oxidized approximately as fast. However, D-lactate is a markedly more effective electron donor for amino acid or sugar transport. Inhibitor studies in which comparisons are made between inhibition of lactose uptake and inhibition of D-lactate oxidation show that the two are qualitatively similar. Anaerobiosis, electron transfer inhibitors such as cyanide, 2-heptyl-4-hydroxyquinoline-N-oxide and amytal, and the specific D-lactate dehydrogenase inhibitor oxamate all effectively block lactose transport. Arsenate and oligomycin have little or no effect on D-lactate-dependent lactose transport (59); amino acid (57) and other sugar transport systems (43) also are not significantly inhibited by these compounds. These observations indicate that the effect of D-lactate on transport is not exerted through production of stable high-energy phosphate compounds. This conclusion is supported by the observation that there is no effect of ATP on transport under conditions in which ATP is accessible to reactive sites within the membrane.

In contrast to the lack of effect on lactose transport by the uncouplers arsenate and oligomycin other compounds which are also uncouplers, e.g., 2,4-dinitrophenol, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and ozide, have a profound effect on transport but do not significantly affect D-lactate oxidation (60). However, the lipid-soluble compounds 2,4-dinitrophenol and CCCP cause leakage postulated to be a result of proton gradient collapse (61). The effect of azide may be

a result of its metal-chelating properties on either electron transport components or other metal-containing proteins which may be either directly or indirectly related to the transport process.

Having established that D-lactate serves as the primary energy source for transport of a number of substrates, Barnes and Kaback (60) undertook to determine whether a separate transport chain for this compound existed and where the energy coupling between D-lactate and the electron transport chain occurred. They found that difference spectra between D-lactate-, succinate-, NADH-, L-lactate- and dithionite-reduced membranes and oxidized membranes are identical (60). Difference spectra between samples reduced by all but NADH furthermore showed no absorption bands. Since cytochrome b_1 reduction rate by these substrates must be directly proportional to their rates of oxidation, these observations indicate that the dehydrogenase for each substrate is coupled to the same cytochrome; there is no unique chain for D-lactate dehydrogenase. Therefore Kaback and coworkers concluded that the site of energy coupling between D-lactate dehydrogenase and transport lies between that dehydrogenase and cytochrome b_1. That succinate can inhibit D-lactate to pyruvate conversion *in vivo* (59,60) but does not inhibit partially purified D-lactate dehydrogenase or the membrane-bound enzyme in the presence of the artificial acceptor dichlorophenolindophenol (DCIP) also indicates that coupling must be proximal to cytochrome. A third line of evidence for this postulate is that the sulfhydryl reagents NEM and PCMB markedly inhibit D-lactate oxidation at the same concentrations which inhibit transport (60). PCMB inhibition is reversible by dithiothreitol. However, these effects do not appear to be mediated at the level of the primary dehydrogenase, as the D-lactate. DCIP reductase activity of

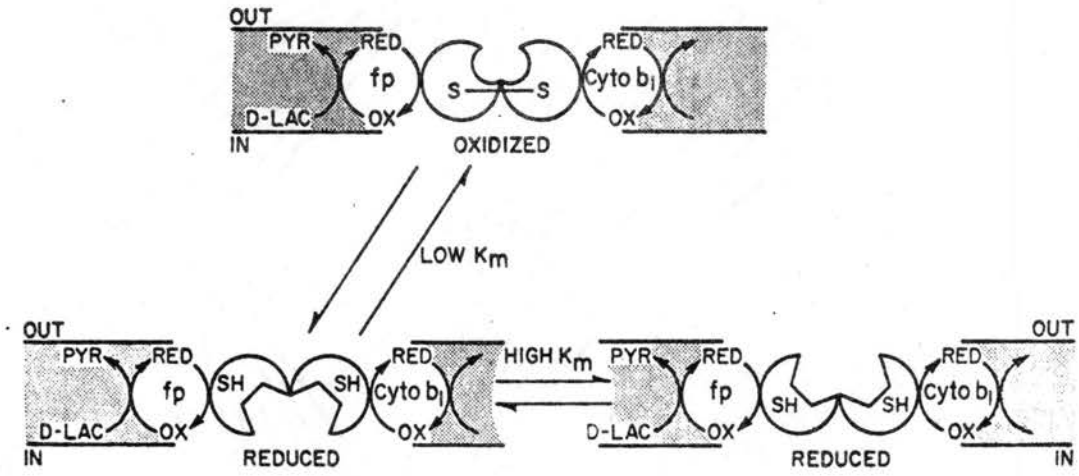
neither vesicles nor partially purified enzyme is sensitive to the sulfhydryl reagents. A diagram of the postulated electron flow is given below.



Temperature response, activation energy and temperature and inhibitor effects on influx and efflux are consistent with the conceptual model for D-lactate dehydrogenase-coupled systems shown in Figure 3 (1). "Carriers" are simplistically shown as electron transfer intermediates which undergo reversible oxidation-reduction. The high affinity oxidized carrier binds substrate on the exterior of the membrane. A critical disulfide in the "carrier" is reduced by electrons coming ultimately from D-lactate, resulting in a conformational change and a concomitant decrease in affinity of carrier for ligand. The ligand is released on the interior surface, and the reduced form of the carrier is oxidized by the electron transport chain, while the electron flow continues to the terminal acceptor oxygen. The reduced form of the carrier may also "vibrate" and catalyze a low affinity, carrier-mediated diffusion process. A conceptual model of the D-lactate dehydrogenase system is shown in Figure 3.

The chemiosmotic coupling theory suggested by Mitchell (62,63) as a possible mechanism for mitochondrial oxidative phosphorylation has been extended to the active transport process in bacteria (64,65). According to the Mitchell hypothesis, oxidative phosphorylation or transport is coupled to a proton or potential gradient. The action of certain

Figure 3. The Kaback D-Lactate Dehydrogenase-Coupled Transport System


OUT
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RED
Cyto b_1
OX
OXIDIZED
LOW K_m
OUT
PYR
RED
fp
D-LAC
OX
IN
SH
SH
RED
Cyto b_1
OX
REDUCED
HIGH K_m
PYR
RED
fp
D-LAC
OX
SH
SH
RED
Cyto b_1
OX
REDUCED
OUT
IN

inhibitors and ionophores mentioned previously can be most readily explained by this model. However, Reeves (66) found that, even though the pH of a suspension decreases on addition of electron donors such as D-lactate, a proton gradient is probably not established under most conditions. He found that in the presence of these donors vesicles do not take up the lipid-soluble weak acid 5,5-dimethyloxozolidine-2,4-dione, which normally diffuses passively across many membranes. Addition of transport substrates had no effect on either rate or amount of acidification. Other studies involving movement of a number of ions led to the same conclusions. Phospholipase-treated membrane vesicles, which retain catalytic activities associated with transport but cannot accumulate solute, exhibit pH changes similar to those seen in untreated vesicles on addition of electron donors and so-called proton conductors such as 2,4-dinitrophenol and CCCP. Since these membranes do not retain their barrier function, it is unlikely that the observed pH changes are a result of proton gradients.

Use of the hydrophobicity probe 1-analino-8-naphthalene sulfonic acid (ANS) has provided insight into membrane structure changes, energy sources and inhibitors (1). A rapid decrease in fluorescence occurs on addition of D-lactate to membrane vesicles in the presence of ANS. The decrease is maintained until anaerobiosis ensues, at which time fluorescence is greatly increased. Relative fluorescence decrease is in the order D-lactate >> succinate ≈ L-lactate. Electron transport inhibitors added before D-lactate greatly inhibit fluorescence changes. When added after D-lactate, inhibitors such as cyanide, HOQNO or amytal, whose site of action is after the site of energy coupling, reverse the initial decrease in fluorescence; inhibitors such as oxamate, NEM and

PCMB which inhibit at or before the coupling site have little or no effect. Phospholipase-treated membranes show qualitatively the same types of fluorescent changes. These studies provide strong support for the intimate involvement of configurational changes in membrane components in the mechanism of active transport.

Transport studies in microorganisms have been numerous and diverse, covering a wide range of compounds and many different microbes. Transport systems for amino acids, carbohydrates, glycolysis and Krebs cycle intermediates, nucleosides, peptides and vitamins have been reported. A long-standing interest in lipoic acid in this laboratory resulted in the investigation of transport of this vitamin and subsequently of other vitamins, including riboflavin and thiamine. The latter compound has been more thoroughly investigated in this laboratory.

Early work by Neujahr on thiamine transport in Lactobacillus fermenti demonstrated the presence of an uptake system which was stimulated by Mg^{++} and glucose and inhibited by iodoacetate. The iodoacetate inhibition was reversed by addition of ATP (67). The pronounced temperature and pH optima, energy dependence, saturation kinetics and stereospecificity observed for the system imply a carrier-mediated, active transport process for thiamine. Accumulation is reversible and is stimulated by ascorbate both uptake and exit are stimulated by K^{+} and by $PO_4^{=}$, exit being stimulated to a much greater extent than uptake with $PO_4^{=}$. Uptake in non-proliferating cells decreases with increasing amounts of thiamine in the growth medium (68).

In phosphate-sufficient cells labeled thiamine is rapidly phosphorylated (69), whereas in phosphate-depleted cells free thiamine comprised 70% of the total, which remained constant. Thiamine is

concentrated 260-fold in L. fermenti. Thiamine-deficient cells showed an increase in thiamine uptake specifically when compared to other types of compounds (70). These cells also showed decreased Mg^{2+}-ATPase activity, and the cellular distribution of the enzyme was altered (71). Preliminary studies with isolated membranes from L. fermenti showed thiamine binding (72).

Kawasaki, et al. (73) found that thiamine uptake in E. coli is energy- and temperature-dependent with a pH optimum of 6.5 and that uptake followed Michaelis-Menten kinetics. The apparent K_m for the process was 8.3×10^{-7} M and the V_{max} was 1.1×10^{-10} moles/min/mg dry weight. There was 175-fold accumulation of thiamine, primarily as thiamine pyrophosphate; at 30 seconds free thiamine comprised 9.2% of the total accumulated, and the thiamine:TPP ratio decreased on further incubation with 1 μM thiamine. However, in the presence of 10 μM thiamine the ratio remained constant.

Kawasaki, et al. (74) isolated a mutant defective in thiamine uptake, yet containing normal levels of thiamine kinase activity. They postulated a model for thiamine transport involving two separate processes, first a specific carrier-mediated passage of free thiamine, and then accumulation by phosphorylation to TPP by the membrane thiamine kinase. Matsuura, et al. (75) found that TPP inhibits both thiamine transport and the thiamine binding protein. Kawasaki and Yamada (76) using E. coli mutants, showed that free thiamine is the compound transported.

Griffith, et al. (77) demonstrated that osmotic shocking of E. coli produced a 50% reduction in the rate of thiamine uptake and released a thiamine binding protein, which was purified and partially

characterized. Shortly afterward Nishimune and Kayashi (78) isolated a thiamine binding protein from E. coli shock fluid and purified it 33-fold. They found that bound thiamine was not exchangeable. Shocked cells retained only a small amount of the thiamine uptake capacity. A third group, Iwashima, et al. (79) also isolated a thiamine binding protein from shocked E. coli cells and purified it 90-fold (80) by affinity chromatography.

Griffith and Leach (81) reported a molecular weight of 40,000, a K_d of 5×10^{-8} M and a pH optimum of 9.0 for the thiamine binding protein. Thiamine pyrophosphate competitively inhibits with a K_I of 5.1×10^{-7} M. Binding is not appreciably affected by Mg^{2+}, phosphate or pyrophosphate, or by variation in temperature between 2 and 37°. Binding is not stimulated by mercaptoethanol or inhibited by NEM, although some inhibition occurs with iodoacetate. Thiamine binding to the binding protein is reversible. Analysis of intracellular radioactive components showed a thiamine:thiamine monophosphate:thiamine pyrophosphate ratio of 2:2:1, and the authors concluded that free thiamine is the accumulated compound.

Since half of the thiamine uptake capacity was retained in the shocked cells, this author pursued the study of the thiamine transport system from the standpoint of examining the activity remaining. The first approach was a study of isolated membranes. Information obtained from membrane perturbation, both chemical and physical, and effects of such perturbation on a membrane activity such as transport, is necessary to supplement information about that activity obtained in other ways. Comparison of membranes isolated from shocked and whole cells might be useful in determining whether basic changes in the transport system occurred during osmotic shock, and if so, whether the activity changes

might be explained in terms of membrane alterations.

Study of various perturbations and their effects on transport in the isolated membranes and in whole cells was envisioned as a method whereby elucidation of the transport system from the standpoint of structure-function relationships might be achieved.

The vocabulary used in transport is at times vague or inconsistent. The terms used in this thesis will be defined as follows. Uptake will apply to any movement of substrate into whole cells or membrane vesicles and will not assume concentration against a gradient. Transport will apply to this same process except that presence of the whole system as it exists in the intact cell is implied. Accumulation will refer only to concentration against a gradient. Equilibrium level and steady-steady level will be used interchangeably to denote the amount of substrate associated in any manner with either membranes or whole cells at a time at which a plateau in uptake has occurred. The term binding will be used only in the strictest sense, implying only interaction between substrate and a transport component molecule. Carrier will be defined in this study as a membrane-bound component which mediates the entry of substrate into the whole cell or membrane. This mediation may involve a soluble, shockable binding protein in the intact cell.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

^{35}S-Thiamine (preparations ranging from 55 to 158 mc/mmole) was obtained from Amersham-Searle. Bovine serum albumin, mercaptoethanol, soybean trypsin inhibitor, phenylmethanesulfonyl fluoride and sodium azide were products of Sigma. Trypsin and lysozyme were obtained from Worthington Biochemical Corp. N-Ethylmaleimide was a product of Eastman Kodak, and thiamine pyrophosphate was from Calbiochem. Chloramphenicol was from Parke-Davis and Company. Dialysis tubing (48 Å pore diameter) was obtained from Arthur Thomas Company. Millipore filters (0.45 and 0.05 mμ) were from Millipore Filter Corporation.

Methods

Growth of Cells

E. coli Crookes cells stored on nutrient agar slants were grown in M-9 minimal medium supplemented with 0.2% glucose (82), unless otherwise specified, on a rotary shaker at 37°.

Osmotic Shock

Cells were grown aerobically in 0.2% glucose M-9 at 37° and were harvested in a continuous flow Sharples refrigerated centrifuge.

Thirty-six liters of early stationary culture yielded about 550 g of cell paste. The cells were shocked by a procedure modified from that of Nossal and Heppel (83).

The cell paste was suspended in about 800 ml of 20% sucrose, 30 mM Tris·HCl, 1 mM EDTA buffer at pH 8.0 at room temperature. This was stirred for 10 minutes at room temperature and then centrifuged at 13,000 x g for 15 minutes. The well-drained pellet was smeared on the inside of a 2 liter Ehrlenmeyer flask, one liter of ice cold 0.5 mM $MgCl_2$ was added and the cells were rapidly suspended by vigorous manual shaking. After standing for 10 minutes, the suspension was centrifuged for 30 minutes at 16,000 x g. The shocked cells were resuspended in 0.03 M Tris, pH 8.0, to a concentration of 1 g cells to 80 ml buffer for subsequent membrane preparation.

Membrane Preparation

Membranes from shocked and normal cells were prepared according to the lysozyme/EDTA method of Kaback (52) except that, since Crookes strain is quite susceptible to the EDTA/lysozyme treatment such that few viable cells remain (less than 1 in 10^3 by plate count on 1% tryptone agar plates), the ultracentrifugation step was omitted. The final preparations were suspended in 0.5 M K-PO_4, pH 6.6, and stored as a number of small aliquots at -15°. Membrane protein was assayed by the method of Lowry, et al. (84), using bovine serum albumin as the standard protein.

Equilibrium Dialysis Studies

Initially binding studies were attempted using the Paulus cell pressure filtration technique (82). This assay method proved unsuccessful

because the membranes greatly retarded flow through the cellulose membranes. Since the amount of thiamine associated with the membranes is a function of relative concentrations of thiamine and membranes, the slowness of the procedure resulted in artifacts arising from changing concentrations. Alternatively, equilibrium dialysis was used as the preferred method of assay.

All equilibrium dialysis studies were done using Chemical Rubber Co. five chamber equilibrium dialysis cells with a cell volume of 1 ml. A single thickness of dialysis tubing (Arthur Thomas, Co., 48 Å pore diameter), cut to the appropriate length and 1 mM EDTA-treated, was used as the dialysis membrane. Equilibrium dialysis suspensions contain 10 mM glucose and 1 mM $MgSO_4$, unless effect of either glucose or Mg^{2+} was being studied, and penicillin/streptomycin to a concentration of 114 µg/ml penicillin/0.1 µg/ml streptomycin. Stirring was accomplished by means of a plastic bead inside each cell and a Chemical Rubber Co. rocker motor, and was allowed to proceed at the desired temperature (usually 22°) for 15-24 hr. Aliquots were taken from each side of the cell, one side containing membranes or protein and ligand and the other side containing ligand only. These were counted in 10 ml Bray's solution in a Packard-Tri-Carb and were corrected by comparison to appropriately quenched standards.

Uptake Studies

Aliquots of the cell suspension were filtered on Gelman Metrical GA-6 filters (pore size 0.45 µ) and washed with the appropriate buffer. The washed cells were suspended in buffer (Tris or phosphate) of the desired concentration and pH, usually 0.01, 0.05 or 0.10 M at pH 6.6

or 8.0. Uptake suspensions contained cells with an A_{620} of approximately 0.1 unless otherwise specified, 10 mM glucose, 1 mM $MgSO_4$, and 0.1 mg/ml chloramphenicol. These were incubated 15-30 min at 37° in a New Brunswick Scientific Co. Metabolyte Water Bath Shaker. At this time ^{35}S-thiamine was introduced to give a thiamine concentration of 1.0 µM unless otherwise specified, and the tube or flask swirled immediately. Aliquots were removed at appropriate intervals and filtered rapidly on Gelman Metrical GA-6 filters on a Bradley multi-cell filtration apparatus. The filters were washed with approximately 1 ml of buffer, air-dried and counted in 10 ml of Bray's solution in a Packard Tri-Carb Liquid Scintillation Spectrometer. All samples were counted to a 1% standard counting error.

Trypsin Treatments

Cells were grown, harvested and suspended as previously described, or aliquots of membrane preparation stored at -15° were thawed and resuspended in appropriate buffer. Trypsin solution at 37° was added to buffered cell or membrane suspensions containing 0.1 mg/ml chloroamphenicol, previously equilibrated to 37°, to give the desired trypsin concentration. The reaction mixtures were shaken gently for 1 hr, at which time a trypsin inhibitor was added, either 2 mM phenylmethanesulfonyl fluoride in water suspension or, preferably, soybean trypsin inhibitor to a concentration of 1.5 times that of trypsin. All additions were made from solutions or suspensions at 37°. The inhibitor/trypsin/cell or membrane suspension was incubated at 37° for 30 min-1 hr. The control was a tube or flask of cells which incubated for the duration of the treatment.

CHAPTER III

MEMBRANES PREPARED FROM NORMAL CELLS

In studying a membrane-associated activity an isolated membrane system may be desirable as a model system. Intracellular components are absent, thereby reducing greatly the ability of the remaining system to metabolize or otherwise compartmentalize products resulting from the membrane activity. Therefore the processes observed in studies using membranes may be limited to the initial step(s) involved in the activity. Isolated membranes represent a derivative of the whole cell membrane and have the added advantage of being arrested in the particular stage of the cell cycle in which the cells existed when removed from growth medium. Therefore, the use of isolated membranes as an approximation to whole cell membranes affords greater simplicity in the system to be studied.

The studies reported in this chapter were done on membranes prepared by the lysozyme-EDTA method of Kaback as described in Methods. This method yields membranes which are essentially devoid of intracellular constituents and consist primarily of intact "unit-membrane" bound sacs, as seen by electron microscopy (52). Since the one essential criterion for any model for the study of transport is semi-permeability, these membranes have been examined carefully for this characteristic. Electron microscopy showed no gross disruption of the membrane surface, and the stain did not penetrate into the interior (52). These membranes behave as osmometers, as demonstrated by light scattering changes on variation

of potassium phosphate concentration. Thus they are capable of accumulation against a gradient, since their barrier capacity remains intact (52).

Time Course of Thiamine Uptake

The kinetics of thiamine uptake in isolated membrane vesicles at pH 6.6 and 8.0 in phosphate buffer is shown in Figure 4. An immediate uptake occurs at both pH's with saturation at approximately 15 minutes. Both curves exhibit an early overshoot followed by re-entry of thiamine to a saturation level of 750 pmoles/mg membrane protein. After an hour uptake commences again to levels somewhat higher than the saturation level. The same uptake patterns were seen with different membrane preparations and in Tris as well as phosphate, although the time at which the second phase of uptake began varied with preparation. Kinetics studies done on several isolated membrane preparations exhibited overshoot at the earliest measurable times (20 seconds).

All membrane suspensions used had an external/internal volume ratio of >1000 as estimated by supernate:pellet volume ratios uncorrected for intra- or inter-membranal volumes. When this volume ratio is taken into account, all preparations tested showed over 200-fold and as much as 1000-fold accumulation, indicating an active transport system. However, a significant amount of uptake occurred also at 4°, as shown in Figure 5. Kinetics studies at 4° and 37° showed no temperature effect for the first 15 minutes. After 15 minutes a linear increase in uptake at 37° occurs, whereas the 4° level remains unaltered. This initial rapid, temperature-independent accumulation most reasonably represents energy-independent binding of thiamine to surface sites (either specifically or

Figure 4. Time Course of Thiamine Uptake at pH 6.6 and pH 8.0 at 25°

Membranes prepared from 4 g of mid-log phase M-9-grown cells and containing less than 0.5% viable cells based on the initial total number of cells were stored overnight at -15° in 0.5 M phosphate, pH 6.6. The membrane suspension was thawed and aliquots were suspended at 25° in standard uptake medium containing 0.05 M phosphate at pH 6.6 (●) or 8.0 (○). Membrane protein concentration was 0.036 mg/ml. Uptake initiation, sampling and counting were done according to the standard procedure and the results plotted as pmoles/mg protein vs. time in minutes.

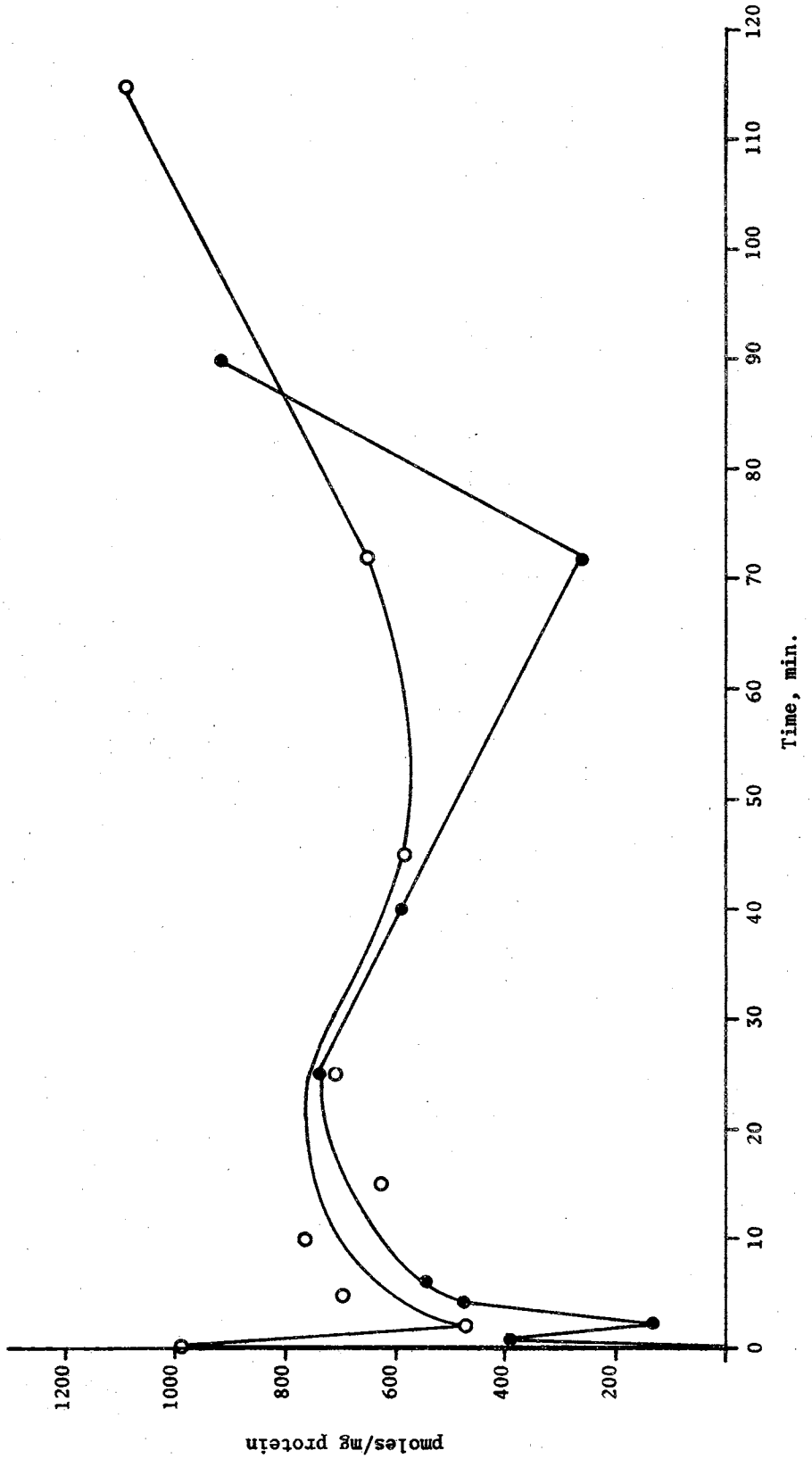
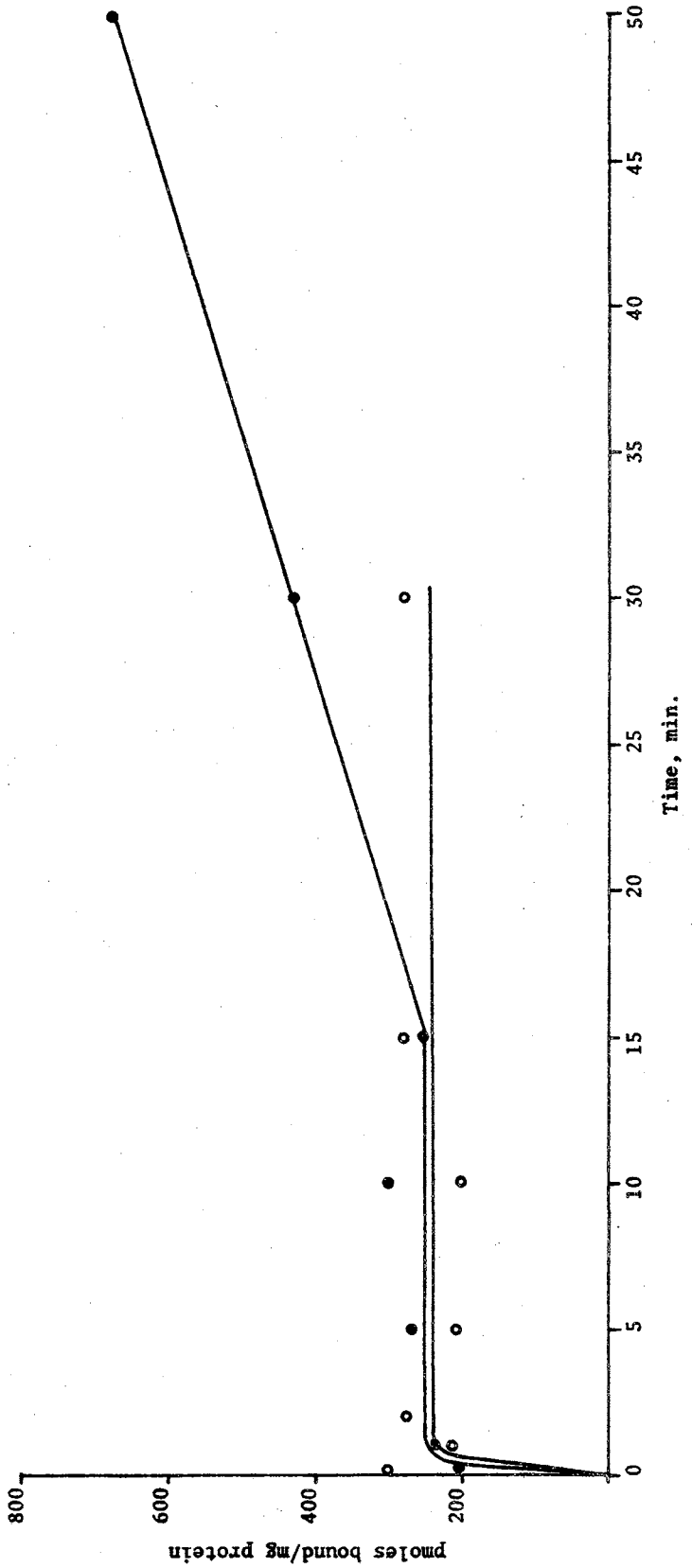

1200
1000
800
600
400
200
pmoles/mg protein
0
10
20
30
40
50
60
70
80
90
100
110
120
Time, min.

Figure 5. Time Course of Thiamine Uptake at 37° and at 4°

Membranes prepared from 3 g of mid-log phase M-9-grown cells and containing 0.34% viable cells based on the initial total number of cells were stored at -15°. A thawed aliquot of these membranes was used to perform uptake studies at 4° (O) and 37° (●) as follows. Two ml of membrane preparation in 0.5 M K-PO_4, pH 6.6, were suspended in 18 ml of water containing the standard assay components for uptake assays. The resulting suspension contained 0.05 M K-PO_4 and a membrane protein concentration of 0.178 mg/ml. Ten ml aliquots were incubated for 45 min in a shaker bath at the two temperatures prior to initiation of uptake. Uptake initiation, sampling and counting were performed in the usual manner and the results plotted as pmoles thiamine/mg protein vs. time in minutes.


pmoles bound/mg protein
800
600
400
200
0
5
10
15
20
25
30
35
40
45
50
Time, min.

non-specifically) without concomitant uptake. Calculations based on the amount of thiamine associated with the membranes per cell, assuming one isolated vesicle/original whole cell (85), give an estimated 100,000 thiamine binding sites per vesicle. This number of sites, whether specific or non-specific, could account for the sizeable amount of thiamine associated with the membrane vesicles even at the lower temperature, at which active transport is thought not to occur. The linear increase after 15 min at 37° then would represent the temperature-dependent active uptake process. Other instances of ligand association with membrane vesicles at low temperature have been cited. Kaback and Deuel (46) found an appreciable amount of proline associated with membranes after 15 minutes incubation at 5°. Lombardi and Kaback (58) found both proline and serine associated with membrane vesicles after 15 minutes at this temperature, both in the presence and absence of energy source. However, more was found in the presence than in the absence of energy source, indicating that there may be a small amount of an energy-dependent process occurring even at this low temperature.

No satisfactory explanation has been found for the lag time, sometimes up to 50 minutes, before initiation of the presumed active process. It is possible that surface alterations of the membrane during vesicle preparation are sufficient to disrupt contact between binding and transport components or between permease and energy coupling system. Lowered temperature has been shown to cause rearrangement, sometimes reversibly, of membrane surface components (86). Incubation of the membranes at the elevated temperature may then re-orient the components, allowing transport to occur. This cold-promoted rearrangement on freezing the vesicles for storage might explain the long lag before initiation of the second

process at 37°.

Effect of Thiamine Concentration on Uptake

Uptake experiments performed on membranes using a thiamine concentration range of 0.02 to 5.0 μM gave the results shown in the double reciprocal plot in Figure 6. At the lower concentrations there is little increase in the amount of thiamine taken up with increasing concentration. An extrapolation of the curve for the low concentrations (0.02 to 0.075 μM) gives an apparent K_m of 2.5×10^{-8} M. At approximately 0.1 μM thiamine there is a sharp break in the curve, and over the higher concentration range a substrate activation effect occurs so that a second K_m cannot be obtained. These results indicate the presence of at least two thiamine uptake systems, one for low concentrations and the other(s) for high concentrations of thiamine.

Effect of Thiamine Concentration on Steady-State Levels of Thiamine

To determine the validity of the use of equilibrium dialysis to circumvent the aforementioned problems encountered with uptake studies, the results obtained using the two methods were compared. Figure 7 shows a double reciprocal plot of thiamine associated with the membrane at equilibrium as a function of thiamine concentration. The results from the two methods are qualitatively similar, since equilibrium studies also suggest the presence of at least two uptake processes. Quantitative differences dependent upon preparation, age of preparation and number of freeze-thaws were evident in double reciprocal plots of uptake and equilibrium experiments. However, a break at 0.10 to 0.15 μM thiamine

Figure 6. Effect of Thiamine Concentration on Uptake

An aliquot of membrane preparation stored at -15° in 0.5 M K-PO_4, pH 6.6, was thawed and resuspended in standard uptake medium to give 0.31 mg/ml membrane protein in 0.1 M K-P, pH 6.6. Uptake initiation was performed according to the standard procedure, and 0.5 ml aliquots were taken at 10 and 20 minutes, filtered and counted. The 10 minute results are plotted by the Lineweaver-Burk method.

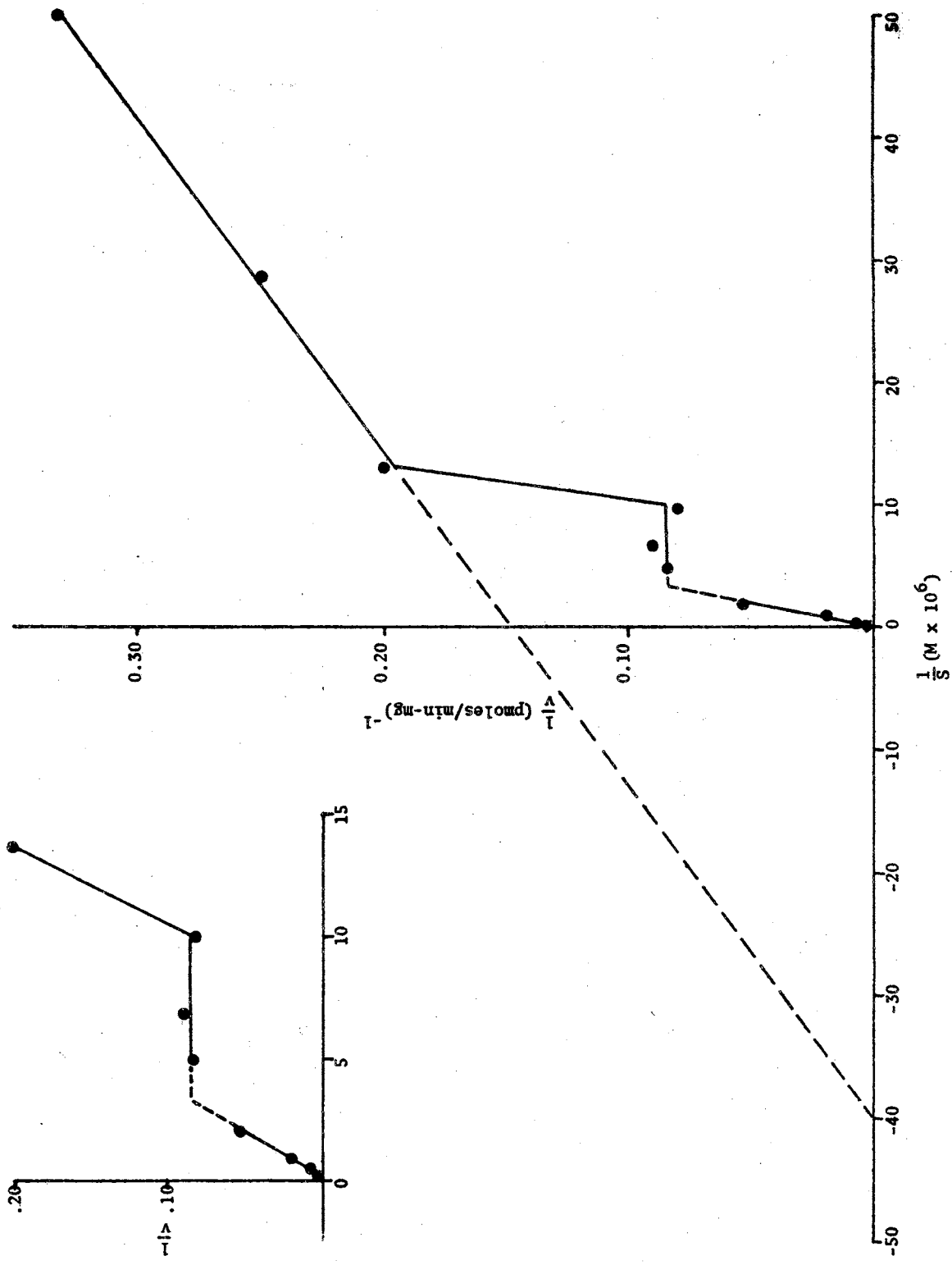
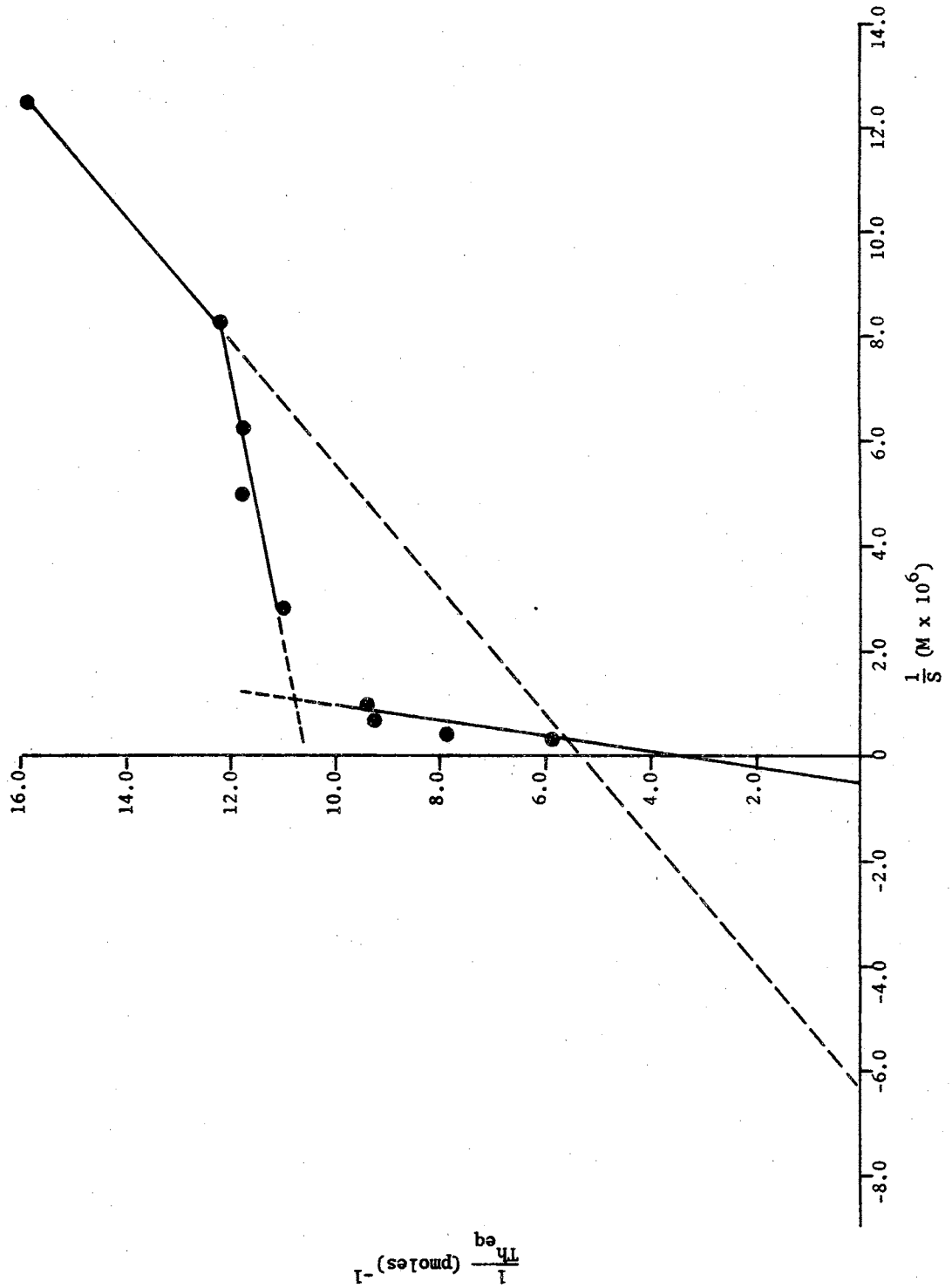

0.30
0.20
0.10
$\frac{1}{v}$ (pmoles/min-mg)$^{-1}$
50
40
30
20
10
0
-10
-20
-30
-40
-50
$\frac{1}{S}$ (M x 10^6)
15
10
5
0
.20
.10
$\frac{1}{v}$

Figure 7. Effect of Thiamine Concentration on Equilibrium Thiamine Levels

Equilibrium dialysis was performed according to the standard procedure at a membrane protein concentration of 0.85 mg/ml over an ^{35}S-thiamine concentration range of 0.02-3.0 μM. The double reciprocal plot shows equilibrium thiamine levels associated with the membranes vs. concentration of added thiamine.


16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
-2.0
-4.0
-6.0
-8.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
$\frac{1}{S}$ (M x 10^6)
$\frac{1}{Th_{eq}}$ (pmoles)$^{-1}$

occurred in all double reciprocal plots; hence it was concluded that the equilibrium method was adequate for studying this complex uptake system in vesicles.

Effect of Energy Source on Equilibrium Thiamine Levels

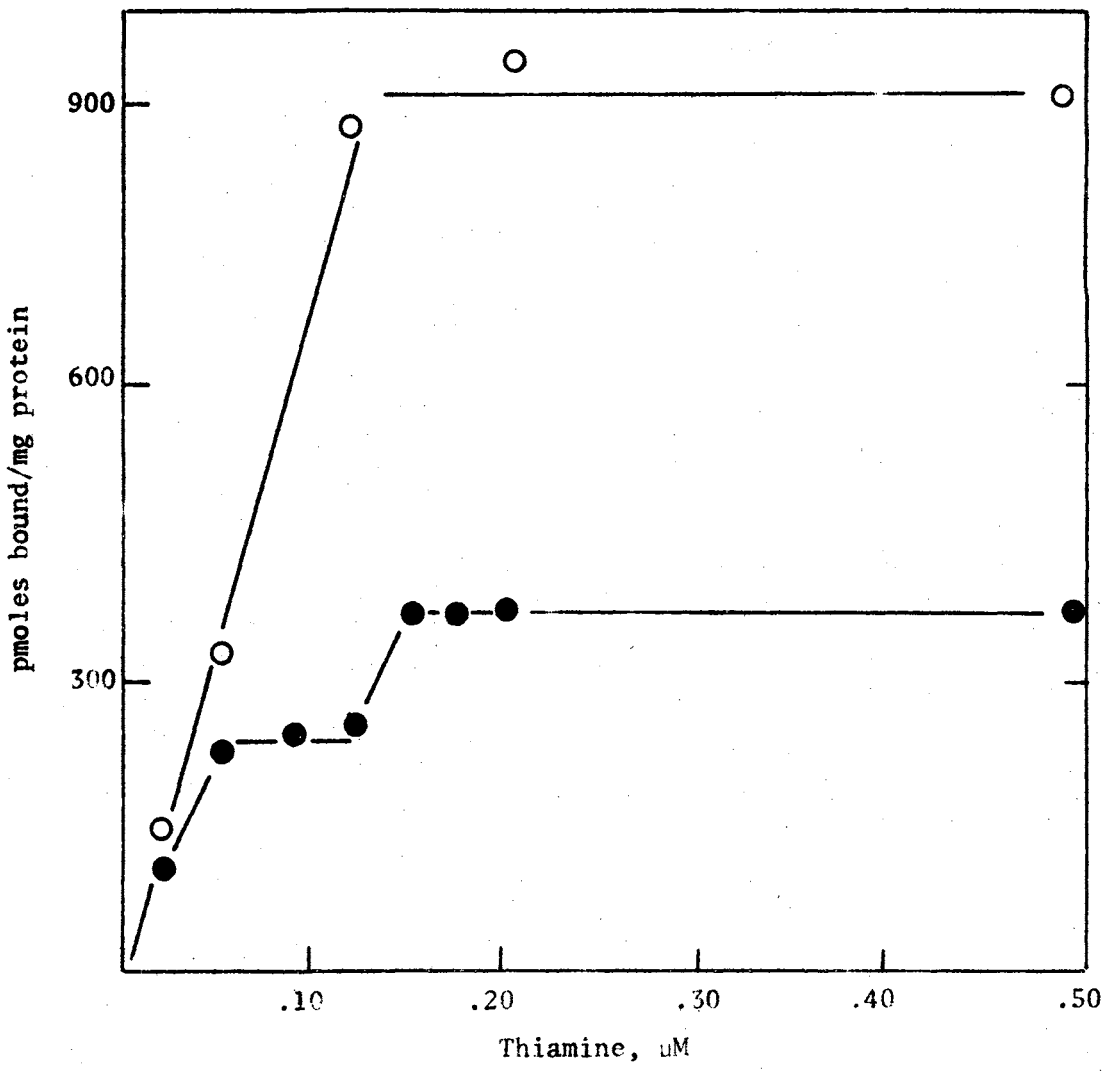
Steady-state levels of thiamine accumulated as a function of thiamine concentration in the presence and absence of energy source are shown in Figure 8. A single saturation occurs in the presence of glucose; the $K_{D\ app}$ for this non-hyperbolic curve is 6.4×10^{-8} M. Omission of glucose decreased the steady-state level of thiamine in this typical experiment by 60%, from 930 to 380 pmoles/mg membrane protein, with a concomitant appearance of a second saturation. The $K_{D\ app}$ for the first saturation process is 2.0×10^{-8} M and for the second is 1.3×10^{-7} M. The second saturation does not extrapolate through the origin. This biphasic phenomenon was noted consistently in the absence of added energy source in normal cell membranes, although the magnitude of the difference in steady-state levels and degree of resolution of the biphasic phenomenon varied from one preparation to the next. These results obtained at equilibrium agree well with the foregoing initial velocity studies in that both suggest the presence of two components or systems for thiamine accumulation dependent on external thiamine concentration. The break in the curve occurs in the same concentration range, i.e., between 0.10 and 0.15 μM.

A number of energy sources were compared with glucose for ability to energize the thiamine transport system in the membrane vesicle. Table I summarizes the results. None of the energy sources was preferable to

Figure 8. Effect of Energy Source on Steady-State Levels of Thiamine

In the presence of glucose: An aliquot of frozen membrane preparation was placed in one side of an equilibrium dialysis cell in the standard assay mixture to give 0.178 mg/ml membrane protein/ml and 10 mM glucose. The dialysis cells were rocked for 16 hr at 25°, and aliquots were counted in the usual manner. The results are plotted as specific activity in pmoles taken up/mg of membrane protein vs. thiamine concentration (O).

In the absence of glucose: The same procedure was followed except that glucose was omitted (●).


900
600
300
pmoles bound/mg protein
.10
.20
.30
.40
.50
Thiamine, uM

glucose in promoting accumulation. D,L-Lactate, the second most effective energy source, promoted accumulation to a level only 5% higher than in the absence of exogenous energy source. Differences in rates of uptake of the various energy sources will not explain the order of preference, since the studies were done at equilibrium.

TABLE I

EFFECT OF ENERGY SOURCE ON EQUILIBRIUM UPTAKE OF THIAMINE

Energy Source (E)	$\frac{\text{Amount taken up in the presence of E}}{\text{Amount taken up in the presence of glucose}} \times 100$
Glucose	100%
D,L-Lactate	46
Glycerol	33
ATP	32
Acetate	30
Succinate	27
None	41

All studies were done under standard conditions using a membrane protein concentration of 0.178 mg/ml and 10 mM energy source. Control values (in the presence of glucose) decreased with time of storage, therefore results are expressed as each substrate's stimulation of accumulation as a percentage of glucose accumulation level.

These studies suggest that there is no obligatory D-lactate dehydrogenase involvement in thiamine uptake in isolated membrane vesicles of E. coli Crookes.

One significant difference between binding curves obtained in the presence and absence of energy source is that omission of energy source resulted in the biphasic phenomenon, whereas the presence of any of the energy sources obliterated the double saturation. This occurred even in the presence of ATP, for which uptake by the membrane is questionable. These observations might suggest that the double saturation seen on omission of energy source results from an uncoupling of energy production in membrane vesicles from the transport system.

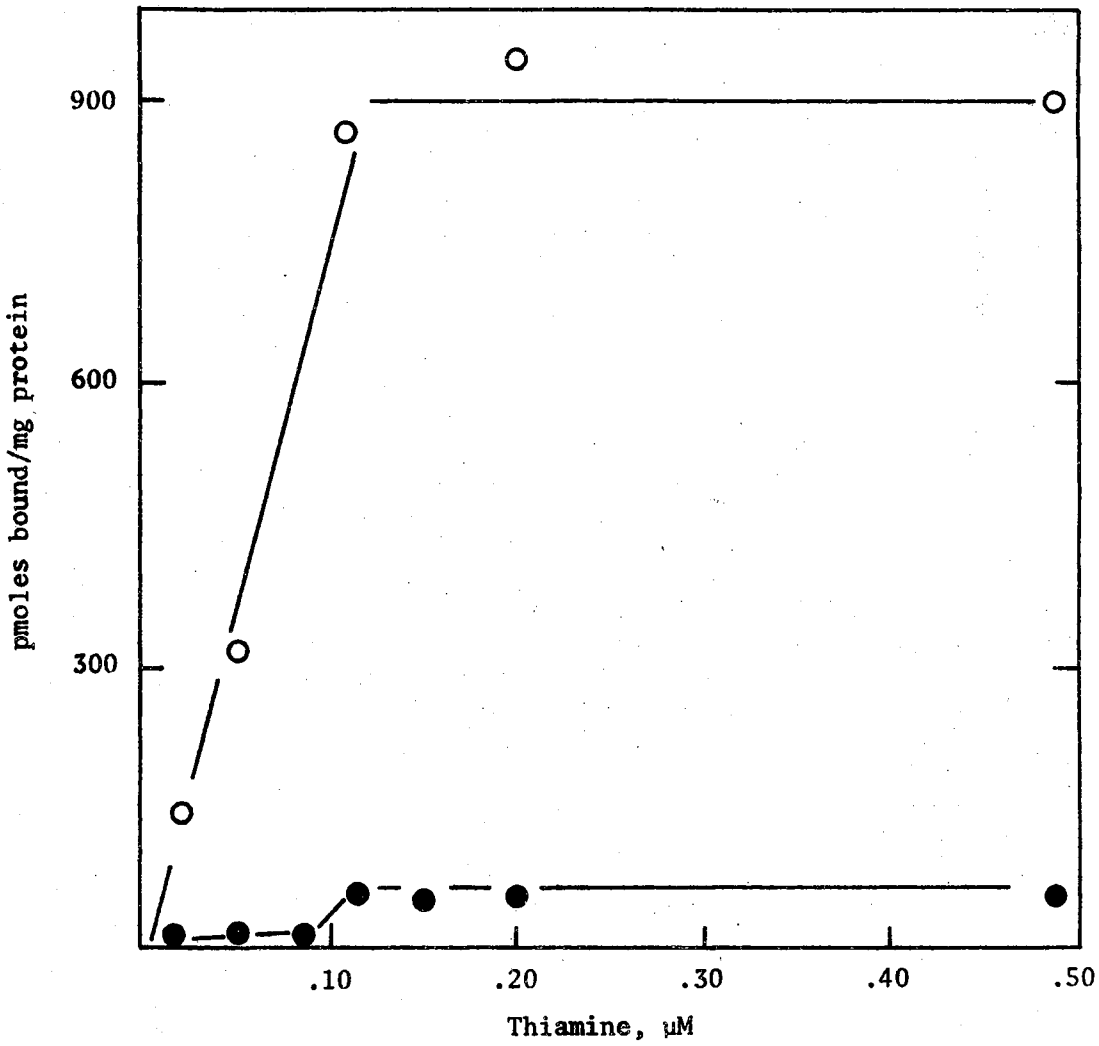
These results show that an exogenous energy source is required for maximal thiamine accumulation in membrane vesicles. Two explanations might account for accumulation of thiamine in the absence of glucose. The first is that uptake may represent only binding to specific components, as previously suggested. The second possibility is that the membranes are not completely depleted of energy source and that part of the uptake is the contribution of an energy-requiring process. It is not possible to attribute the activity of the membranes in the absence of energy source to either explanation on the basis of energy source omission studies alone.

Effect of Inhibitors on Steady-State Thiamine Uptake

Additional evidence for energy dependence of a cellular process is derived from studies using inhibitors such as cyanide, azide, 2,4-dinitrophenol and fluoride, which block energy production. Figure 9 shows the effect of 10 mM azide on equilibrium thiamine levels. Thiamine uptake is almost completely obliterated, the membranes retaining only 5% of the steady-state level of the control. Even at this low level of

Figure 9. Effect of Azide on Equilibrium Thiamine Accumulation

The conditions for this experiment were identical to those for the experiment represented in Figure 8, except that the membranes were incubated with 10 mM azide in the standard assay medium without glucose for 20 min prior to the addition of glucose and thiamine. No azide (O), 10 mM azide (●).


900
600
300
pmoles bound/mg protein
.10
.20
.30
.40
.50
Thiamine, μM

activity a double saturation is evident. Uptake in the absence of energy source is 7.5 times that in the presence of azide and 42% of that in the presence of glucose. If the equilibrium level of thiamine in the absence of glucose is solely the energy-independent binding of ligand to a specific binding component or components, these results might indicate that 10 mM azide almost completely inhibits binding to the component(s). Tsay, et al. (87) reported an inhibitory effect of azide on a glycerol binding component in shock fluid from Pseudomonas aeruginosa. A similar study on a partially purified thiamine binding protein preparation from E. coli Crookes showed no significant inhibitory effect of azide at a concentration of 10 mM. At 0.10 μM thiamine 660 pmoles/mg membrane protein were bound in the absence of azide, whereas 610 pmoles/mg were bound in the presence of the inhibitor, giving a negligible 8% inhibition at an azide concentration which almost completely inhibits uptake in vesicles. This fact suggests that the effect seen on uptake in the membrane is on a component of the thiamine transport system other than the binding protein, or that the thiamine binding protein exists in an azide-affected conformation only in the membrane matrix. The latter explanation assumes that at least part of the activity remaining in the isolated membrane is attributable to residual thiamine binding protein, which may not be the case.

Alternatively, it might be concluded that the membranes in the absence of glucose are not completely depleted of energy source. Azide addition might then uncouple energy production from the transport process, such that only binding to surface sites is seen.

Cyanide produced the same inhibitory effect at a concentration of 10 mM (Table II). However, no biphasic phenomenon was observed in the

presence of cyanide. Fluoride at 1 mM produced a biphasic curve and a concomitant 35% decrease in equilibrium thiamine levels. A concentration of 10 mM fluoride almost completely inhibited activity (Table II).

TABLE II

EFFECT OF INHIBITORS ON THIAMINE STEADY-STATE PARAMETERS

Inhibitor	Concentration mM	K_{d_1} x 10^8 M	K_{d_2} x 10^7 M	% Inhibition of Equilibrium Thiamine Levels	
				Saturation 1	Saturation 2
None	----	4.5	----	0	0
NaN_3	10	2.0	1.3	94	91
NaCN	10	7.0	----	--	97
KF	1	3.5	1.09	35	51
	10	~4	1.05	97	88
DNP	10	4	----	--	15
Oxamate	10	4.3	----	--	-20
	Ratio TPP/Thiamine				
TPP	1:1	2.4	1.43	40	0
	10:1	~4	~1.3	98	95

All experiments were performed under standard conditions of equilibrium dialysis. Inhibitors were incubated for 15 min with the suspensions before the addition of glucose and thiamine.

In contrast to the inhibition of accumulation by the electron transport and metabolic inhibitors discussed above, the uncoupler 2,4-dinitrophenol at 10 mM gave a negligible 15% inhibition in the isolated membranes. This observation could be explained by the arguments put forth by Barnes and Kaback (59,60). They postulated requirement for an operable electron transport chain for induction of a conformational change in the carrier protein, allowing subsequent binding and transport. If the chain is blocked, the oxidation-reduction cycle is broken, precluding binding and transport (60). On the other hand, in the presence of oxidative phosphorylation inhibitors such as 2,4-dinitrophenol electron transport remains functional; therefore binding and transport are not affected. Alternatively, if the system were energized by the PEP phosphotransferase system, no inhibition would be expected, since uncouplers of phosphorylation stimulate the production of PEP (29). A summary of inhibitor data is given in Table II.

More specific information concerning the thiamine transport system and its energization in membrane vesicles is obtained using competitive inhibitors. To ascertain whether D-lactic dehydrogenase might be involved in transport of thiamine the effect of the specific D-lactate dehydrogenase inhibitor oxamate on thiamine accumulation was determined. Rather than an inhibitory effect of the compound, there was a 20% stimulation of thiamine accumulation. This result reinforces the suggestion made on the basis of D,L-lactate's inefficacy as an energy source that the thiamine transport activity remaining in the isolated membrane is not dependent on the D-lactate dehydrogenase.

Thiamine pyrophosphate (TPP) is a competitive inhibitor for the thiamine binding protein (75,77) and for thiamine transport (75). At

equimolar concentrations of thiamine and TPP a biphasic curve was obtained. A 40% reduction in equilibrium thiamine levels occurred at thiamine concentrations between 0.05 and 0.10 μM, but inhibition above 0.20 μM was insignificant. A ten-fold excess of TPP decreased thiamine accumulation by 95% at equilibrium.

The unequal effects of TPP at lower TPP:thiamine ratios on the lower and higher thiamine concentration ranges might suggest that there are two components or sites, one of which is accessible to the surface and available for binding TPP, and the other unexposed and unavailable until the first component is saturated. The higher TPP:thiamine ratio might saturate the first component, with a concomitant exposure of the second site, such that both thiamine-binding components are inhibited.

Involvement of Divalent Cation in Thiamine Accumulation

The Mg^{2+} requirement by the thiamine uptake system is illustrated in Figure 10. In the absence of M^{2+} there is an 80% reduction in the maximal steady-state level of thiamine. Addition of 10 mM EDTA produces a greater reduction with the appearance of a double saturation in the binding curve. Addition to the EDTA-treated membranes of Mg^{2+} to a concentration of 15 mM, giving an effective concentration of 5 mM Mg^{2+}, restored activity to two-thirds of the level of the control containing the standard 1 mM Mg^{2+}; concomitantly the first saturation was obliterated. At low thiamine concentrations almost complete restoration occurred. This selective effect of Mg^{2+} on one of the saturations might suggest that the two saturations represent different components or systems which are involved in thiamine uptake. Ca^{2+} did not replace Mg^{2+}

Figure 10. Involvement of Divalent Cation in Accumulation of Thiamine

Membrane vesicles were prepared by the usual method except that after spheroplast formation the centrifuged cells suspended in 0.1 M K-PO_4/10 mM $MgSO_4$ were incubated for 20 min in an ice-salt bath and sonicated by three 10 sec bursts on the Branson Sonifier.

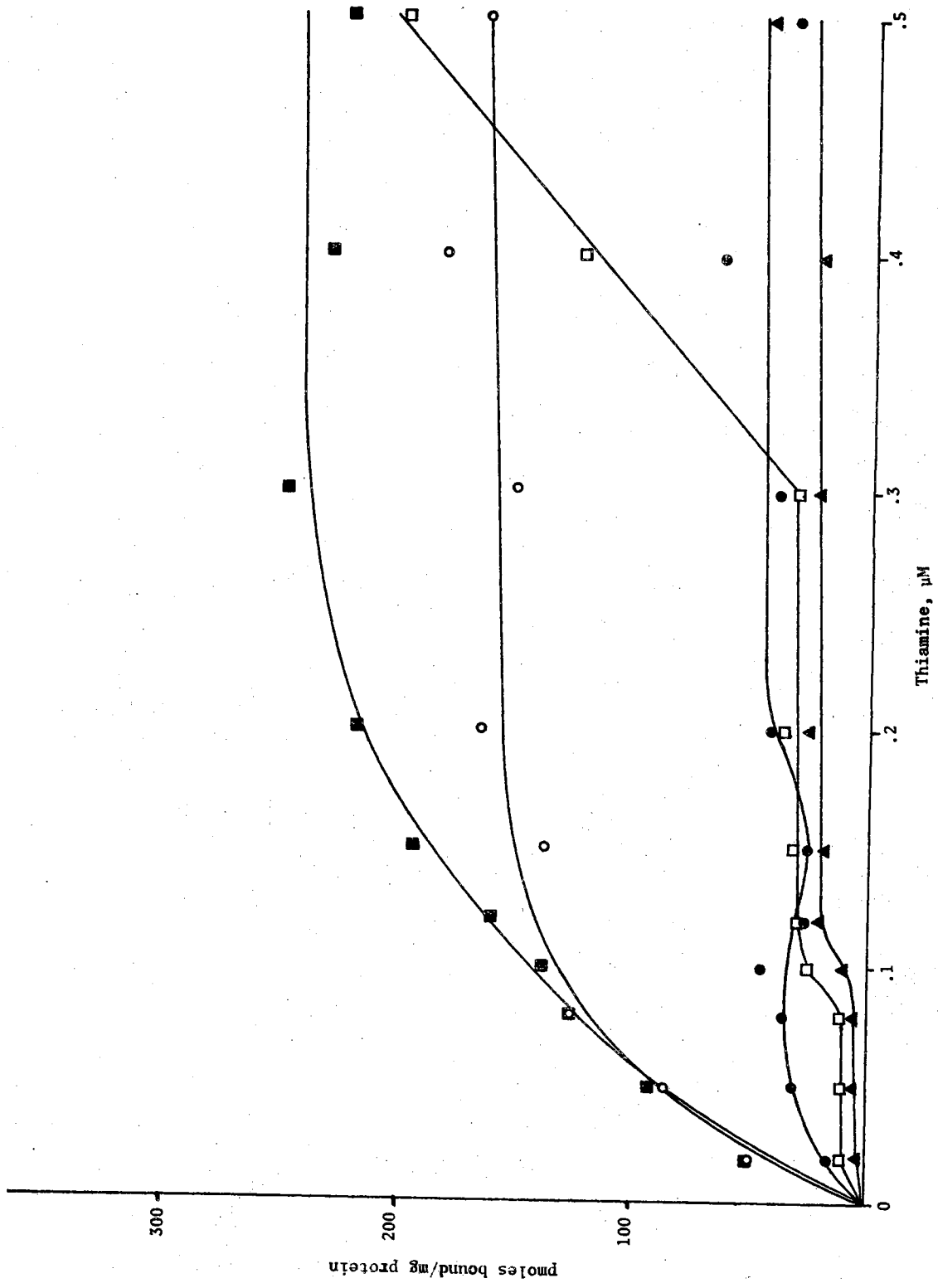
Presence of Mg^{2+}: Equilibrium dialysis cells were prepared in the usual manner and contained 10 mM Mg^{2+}. Concentration of protein was 0.22 mg/ml for all studies in this experiment(■).

Absence of divalent cation: Dialysis cells were prepared in the usual manner except that divalent cation was omitted(●).

Presence of EDTA: To the usual equilibrium dialysis mixture was added 10 mM tetrasodium EDTA rather than divalent cation(△).

Presence of Ca^{2+}: 10 mM $CaSO_4$ replaced $MgSO_4$ in the usual mixture. These were rocked for 16 hr at 25°, and 0.5 ml samples were taken for counting in the usual manner(□).

EDTA-treatment, Mg^{2+}-reconstitution: After sampling of the EDTA-treated membranes, 7.5 µl aliquots of 1.0 M $MgSO_4$ were added to each compartment to give a 1.5-fold excess of Mg^{2+} over EDTA, or an effective Mg^{2+} concentration of 5 mM. Dialysis was continued for another 16 hr at 25°, at which time 0.3 ml aliquots were counted(○).


pmoles bound/mg protein
300
200
100
0
.1
.2
.3
.4
.5
Thiamine, µM

in restoring uptake activity at thiamine concentrations up to 0.30 μM and also effected a double saturation. At the higher thiamine concentrations an increase approaching the steady-state level of the Mg^{2+}-containing control appeared. This Ca^{2+} effect was not pursued because of the anomalous effects this cation is known to have on membrane structure.

pH Effect

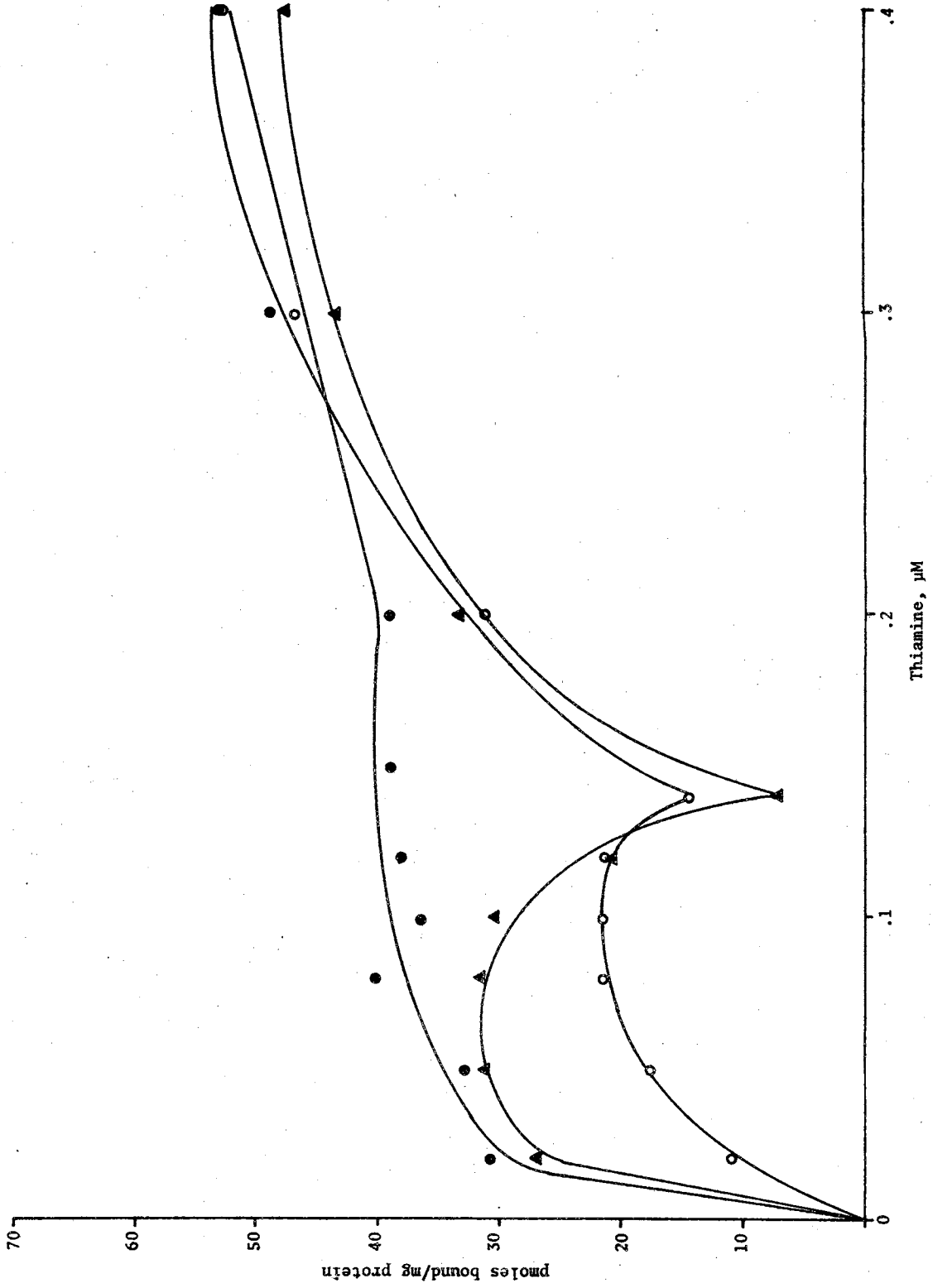
The effect of pH on steady-state thiamine levels is shown in Figure 11. Increasing the pH from 6.6 to 7.8 or above produces a pronounced double saturation and a depressed transport capability at concentrations a little less than those necessary for visualization of the second saturation. At pH 7.8 there is a slight decrease in the first saturation level, with a sharp drop to 18% at 0.15 μM thiamine, compared to levels at pH 6.6. At pH 9.0 only 60% of the pH 6.6 saturation level is bound, with a decrease to 36% at 0.15 μM. The effect of pH varied quantitatively from one membrane preparation to another, but the general effect was the same in that all showed a marked decrease in the 0.10-0.15 μM thiamine range at higher pH. Accumulation over the second saturation concentration range, i.e., concentrations above 0.15 μM, was relatively independent of pH. These results tend to reinforce the idea of a two-transport system capability of this organism for thiamine, one being pH-dependent, the other pH-independent over the pH range studied.

Effect of Concentration of Membrane on Equilibrium Thiamine Levels

Thiamine steady-state levels in membranes from unshocked cells are a linear function of membrane concentration at thiamine concentrations of

Figure 11. Effect of pH on Steady-State Levels of Thiamine

Freeze-thawed membrane preparation was added to the standard equilibrium dialysis mixtures in 0.5 M K-PO_4 at pH 6.6 (●), 7.8 (▲), and 9.0 (○). After 16 hr at 25° sampling and counting were done in the usual manner and the results plotted as pmoles bound/mg protein vs. thiamine concentration.


pmoles bound/mg protein
70
60
50
40
30
20
10
0
.1
.2
.3
.4
Thiamine, µM

0.10 and 0.50 μM, the concentrations at which the two saturations occur (Figure 12).

Effect of Trypsin Digestion of Membranes on Equilibrium Levels of Thiamine

To determine the accessibility of transport components to the membrane surface the effect of trypsinization on isolated membranes was studied. Table III shows the effect of various conditions of trypsin treatment on equilibrium thiamine levels over a thiamine concentration range of 0.02-0.50 μM. Trypsinization for 30 minutes at 30° causes slightly decreased (∿ 11%) thiamine levels at thiamine concentrations less than 0.08 μM. However, at concentrations greater than 0.08 μM a pronounced increase (30%) in the amount of thiamine associated with the membrane resulted. Trypsin treatment for 60 minutes at 30° gave no more difference at the lower thiamine concentrations, but the levels at the higher concentrations were decreased from the half-hour levels.

These results may indicate that a component or site of a system for accumulation at the lower thiamine concentrations exists at the surface in an arrangement such that it is available to thiamine and only moderately available to trypsin. On the other hand, a second component or site for accumulation at higher concentrations is partially buried within the membrane, such that trypsinization either exposes the buried sites or causes a conformational alteration exposing this site or component. Further trypsinization then causes degradation of this exposed site.

This conclusion is substantiated by results of trypsinization for 40 minutes and for 1 hour at 37° rather than 30°. The 40 minute treatment

Figure 12. Effect of Membrane Concentration on Steady-State Levels of Thiamine

Membrane preparation prepared in the usual manner and stored frozen, was thawed and added to equilibrium dialysis cells containing the standard assay mixture to give membrane protein concentrations ranging from 0.12 to 1.23 mg/ml. ^{35}S-Thiamine concentrations used were 0.10 (O) and 0.50 (●) μM, the concentrations representing the two saturations under conditions resulting in a double saturation. After rocking 16 hr at 25°, the dialysis cells were sampled in the usual manner for scintillation counting. The equilibrium thiamine levels in pmoles are plotted against concentration of membrane protein in mg/ml.

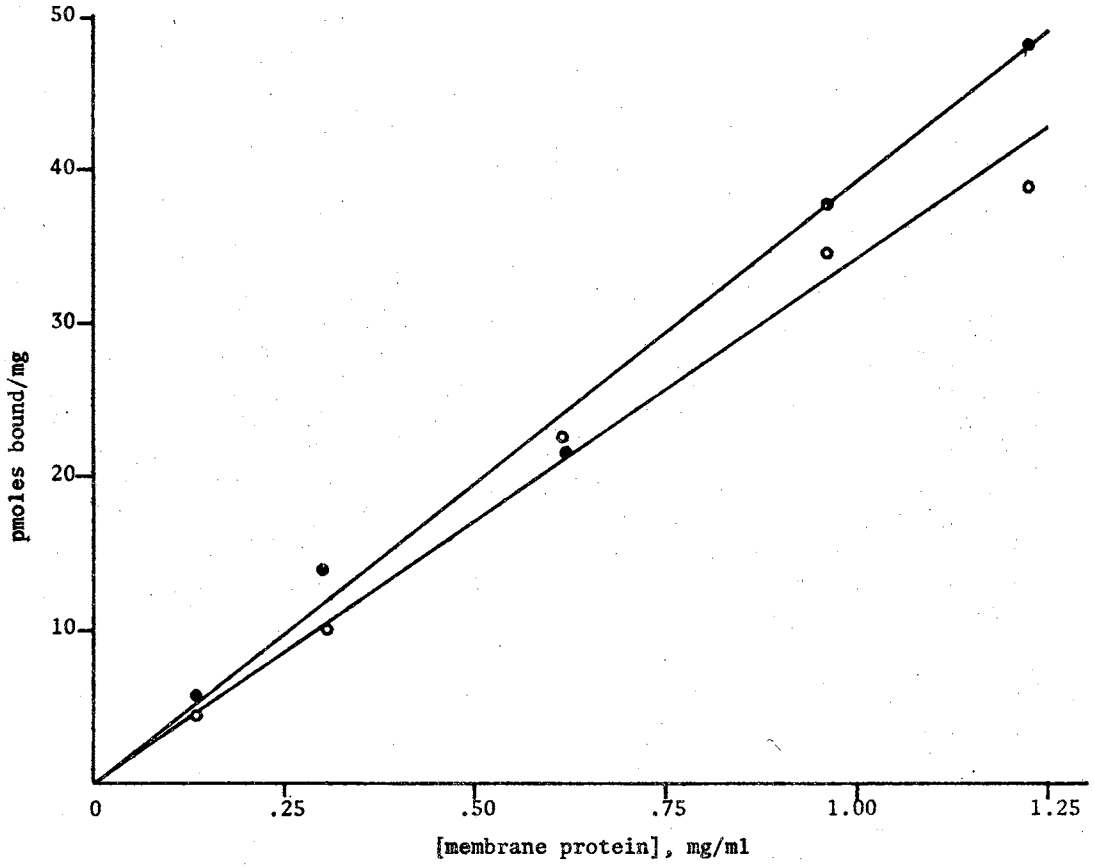

50
40
30
20
10
pmoles bound/mg
0
.25
.50
.75
1.00
1.25
[membrane protein], mg/ml

TABLE III

EFFECT OF TRYPSINIZATION ON EQUILIBRIUM THIAMINE LEVELS

	Buffer	Thiamine Accumulation at Equilibrium pmoles/mg protein	
		Saturation 1	Saturation 2
Control[a]	$PO_4^{=}$	410	515
30 min.		565	680
60 min.		435	502
Control[b]	$PO_4^{=}$	702	780
40 min.		748	875
Control[c]	$PO_4^{=}$	196	172
60 min.		108	115
Control[d]	$PO_4^{=} + Ca^{2+}$	14	67
20 min.		37	62
Control[e]	Tris	94	176
30 min.		147	162

All trypsinization experiments employed 1 mg/ml trypsin and 2 mM PMSF. Controls containing all components except trypsin and PMSF were incubated at the temperature for trypsinization for the duration of the treatment. Assays were performed by the standard equilibrium dialysis method.

[a]In 50 mM potassium phosphate, pH 7.8, at 30°; [membrane protein] = 0.178 mg/ml.

[b]In 50 mM potassium phosphate, pH 8.1, at 30°; [membrane protein] = 1.78 mg/ml.

[c]In 50 mM potassium phosphate, pH 7.8, at 37°; [membrane protein] = 0.50 mg/ml.

[d]In 50 mM potassium phosphate, pH 7.8, containing 10 mM Ca^{2+} at 30°; [membrane protein] = 1.05 mg/ml.

[e]In 50 mM Tris, pH 8.0, at 30°; [membrane protein] = 0.178 mg/ml.

increased thiamine levels over the higher concentration range and had little effect below 0.10 μM thiamine. Treatment for 1 hour at the elevated temperature caused a reduction in thiamine levels at both sites due to enhanced degradation of the membrane.

The surface localization of thiamine transport components is influenced by the buffer employed. In contrast to the results seen in Table III for the phosphate buffer somewhat different results are obtained in the presence of Tris. Accumulation in untreated membranes is much less in Tris than in phosphate. Under identical conditions trypsinization for 30 minutes of membranes suspended in Tris buffer enhances accumulation at all thiamine concentrations. This buffer apparently causes a membrane alteration such that the first component (or site) as well as the second is less accessible to thiamine. Trypsinization then uncovers both sites, making them more available to the ligand.

Earlier studies on the effect of divalent cations showed an anomalous effect by Ca^{2+} (Figure 10). Trypsin studies in the presence of Ca^{2+} shed some light on the behavior of this ion on the membrane with respect to thiamine transport components. The pronounced double saturation and lowered accumulations were evident in the control; trypsinization effected a 240% increase in thiamine levels at 0.08 μM with a slight decrease (10%) in the levels at the higher concentrations. These results suggest that Ca^{2+} causes a membrane alteration or specific protein conformation change which buries the higher affinity site. Trypsinization then alters the membrane, exposing the site to the surface and enhancing availability to ligand. Increasing the thiamine concentration above 0.30 μM overrides the Ca^{2+} effect, exposing the second site to the surface, thereby enhancing ligand association with it.

Effect of Physical Perturbation of Membranes on Steady-State Thiamine Accumulation

Storage of isolated membranes at -15° alters the thiamine uptake system in the vesicles. As shown in Figure 13, the saturation which occurs at higher thiamine concentrations disappears and that at the lower concentrations appears concomitantly on aging of membrane preparations at -15° in 0.5 M K-PO_4 buffer, pH 6.6. Figure 13 shows the changes in the binding curves at three time intervals, and Figure 14 summarizes these results for a 40-day period. This phenomenon manifests itself as a gradual lowering of K_d for the saturation with a concomitant loss of binding and/or accumulation ability. The results using this particular membrane preparation are seen typically in membranes prepared from untreated cells.

Storage at -15° apparently causes a membrane alteration which is reflected in transport capability. One explanation for this observation would be movement of one transport component of a two-transport system away from the surface and concomitant exposure of the second component. Alternatively, one molecule with two thiamine binding sites with differing affinities for thiamine may undergo the same type of membrane orientation alteration. The latter seems a more reasonable explanation, since the disappearance of equilibrium thiamine level at the higher concentrations coincides with appearance of thiamine uptake at the lower concentrations.

Table IV shows that glycerol (20%) stabilizes membranes at -15° overnight in the presence of 10 mM EDTA, which normally drastically reduces thiamine accumulation, as shown in Figure 10.

Sonication of membrane preparations also decreased accumulation

Figure 13. Effect of Storage of Membrane Preparations at -15° on Equilibrium Thiamine Levels

Binding curves for experiments performed under standard conditions using frozen membrane preparation 1 (●), 8 (○) and 23 (▲) days after preparation are shown.

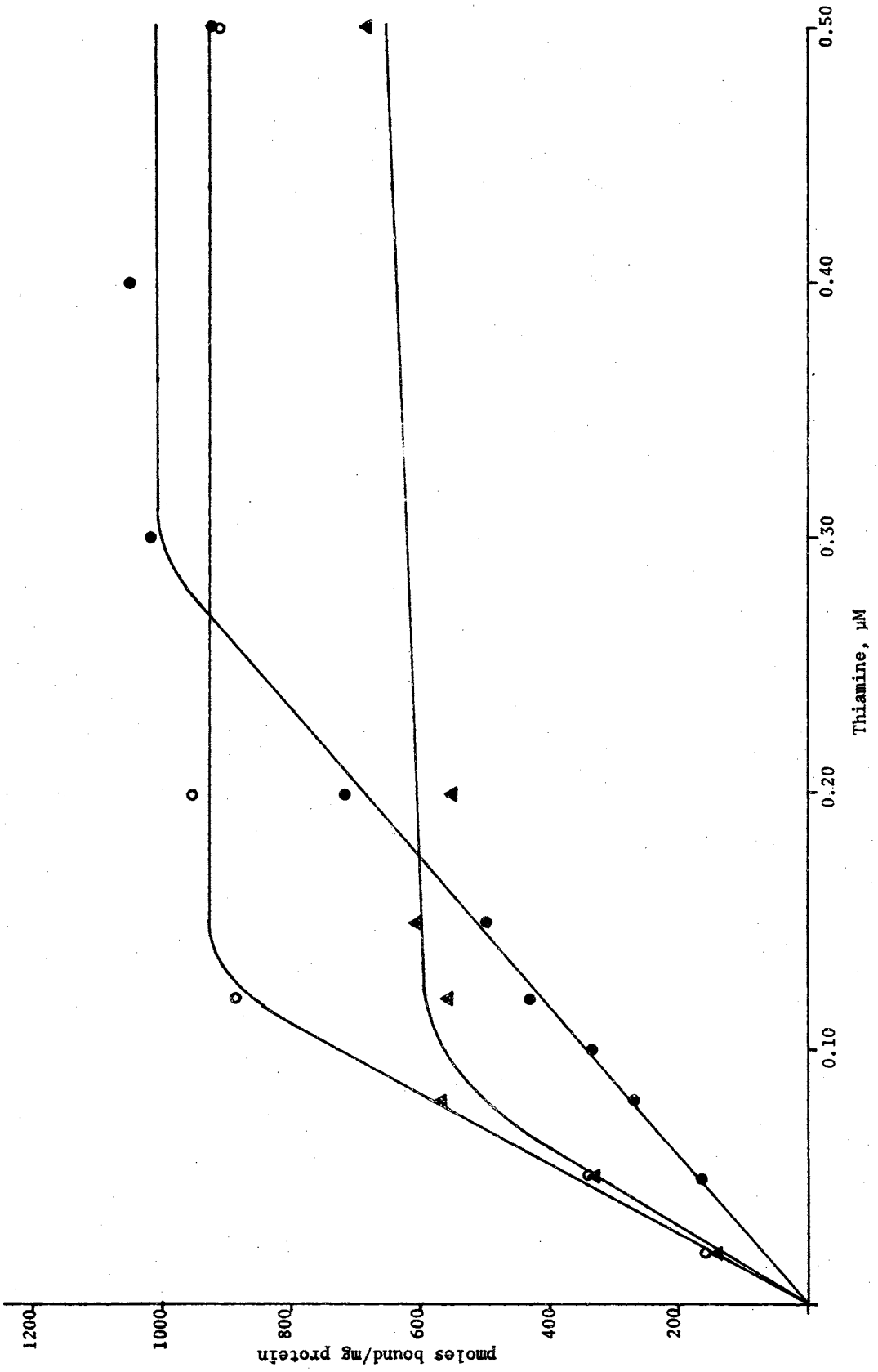
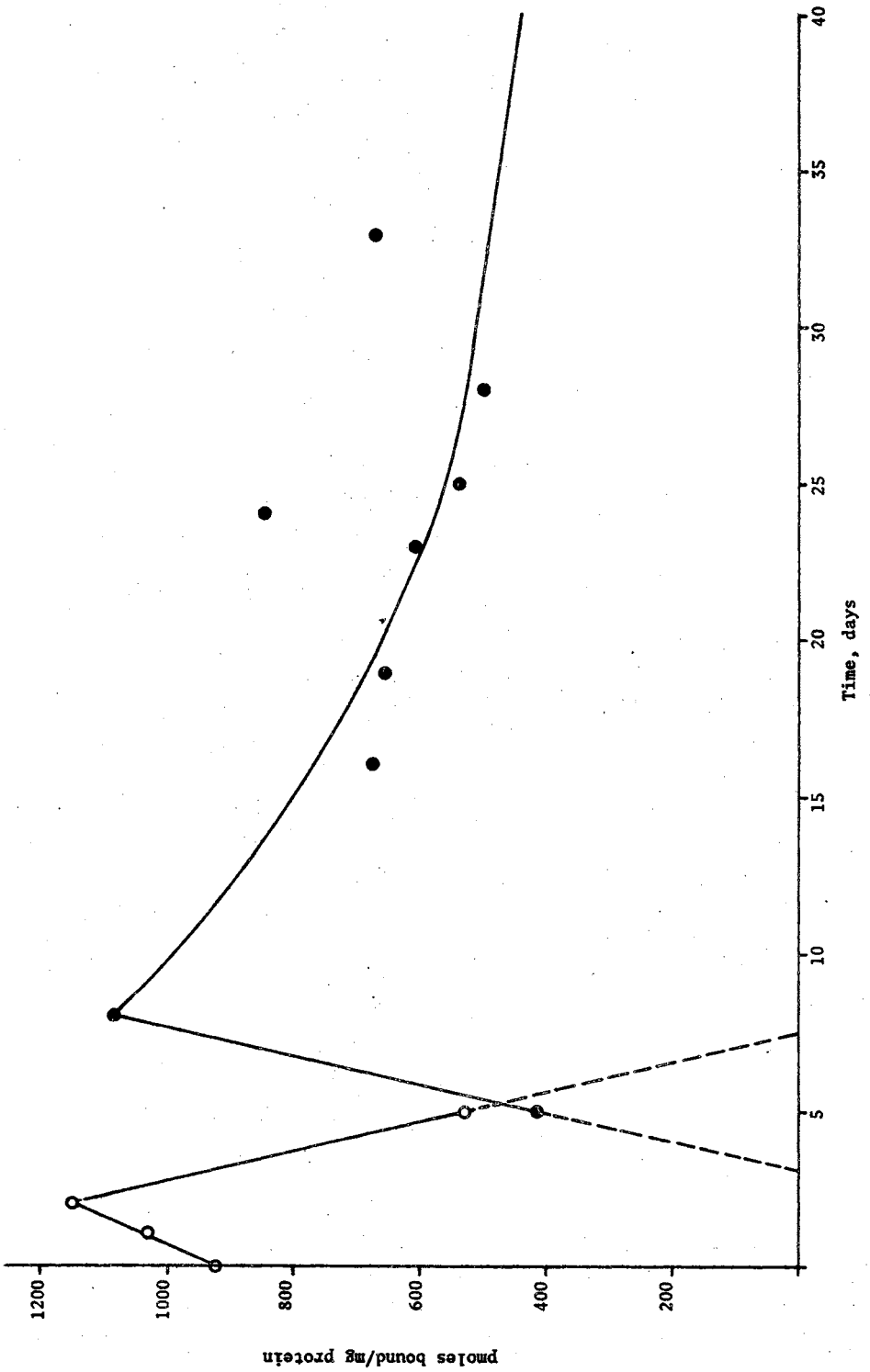

pmoles bound/mg protein
1200
1000
800
600
400
200
0.10
0.20
0.30
0.40
0.50
Thiamine, µM

Figure 14. Effect of Aging on Accumulation at 0.15 and 0.45 μM Thiamine

The equilibrium thiamine levels in pmoles/mg protein at 0.15 (O) and 0.45 (●) μM thiamine, concentrations at which the two saturations are seen under the appropriate conditions, are plotted using data from experiments run under standard conditions over a 43-day period.


pmoles bound/mg protein
1200
1000
800
600
400
200
5
10
15
20
25
30
35
40
Time, days

ability. Results are shown in Table V for two preparations, one which exhibited a high accumulation capacity, and another which accumulated relatively small amounts of thiamine. Sonication of the higher activity preparation in 20% glycerol produced a second saturation with no significant change in K_{d_2} but with a 17-fold decrease in the amount of thiamine accumulated. Sonication of the lower activity preparation under essentially identical conditions decreased 7-fold the ability of the membranes to accumulate thiamine. To determine whether oxidation of critical groups such as sulfhydryl groups might be responsible for the loss of activity, a parallel sample containing 10 mM mercaptoethanol as well as 20% glycerol was sonicated simultaneously. There was no appreciable stabilization in the presence of mercaptoethanol. Sonication must produce either osmotically insensitive vesicles or membrane fragments which retain the capacity to bind but not accumulate thiamine.

TABLE IV

STABILIZATION BY GLYCEROL ON STORAGE AT -15°

Treatment	K_d x 10^7	$Thiamine_{max}$ pmoles/mg protein
None	1.62	379
20% Glycerol, 10 mM EDTA	1.1	320

Membranes prepared in the usual manner were divided into two aliquots. One was suspended in 0.5 M $K\text{-}PO_4$, pH 6.6, and the other in the same buffer to which 20% glycerol and 10 mM EDTA had been added. Both were stored overnight at -15° and assayed for binding in the usual manner.

TABLE V

EFFECTS OF SONICATION

Treatment	K_{d_1}	K_{d_2}	$Thiamine_{max_1}$	$Thiamine_{max_2}$
	x 10^8M	x 10^7M	pmoles/mg protein	pmoles/mg protein
High Activity Membranes				
None	---	1.62	---	379
20% Glycerol	2.2	1.68	10	22
Low Activity Membranes				
None	5.7	----	33	---
20% Glycerol	∿4	----	6	---
20% Glycerol, 10 mM HSEtOH	∿4	----	8	---

Sonication was performed on membranes suspended in 0.5 M K-PO_4, pH with and without glycerol and mercaptoethanol for 40 minutes at < 10° using a Raytheon sonicator.

CHAPTER IV

MEMBRANES PREPARED FROM SHOCKED CELLS

Since osmotically shocked E. coli cells retain half of their thiamine uptake ability after release of a thiamine binding protein (77), a study of the activity remaining in the shocked cells was undertaken. Membranes were prepared by the same procedure as for unshocked cells (52). Surface differences between membranes prepared from shocked and unshocked cell membranes were apparent; shocked cell membranes appeared more adhesive and were dispersed into suspension with difficulty.

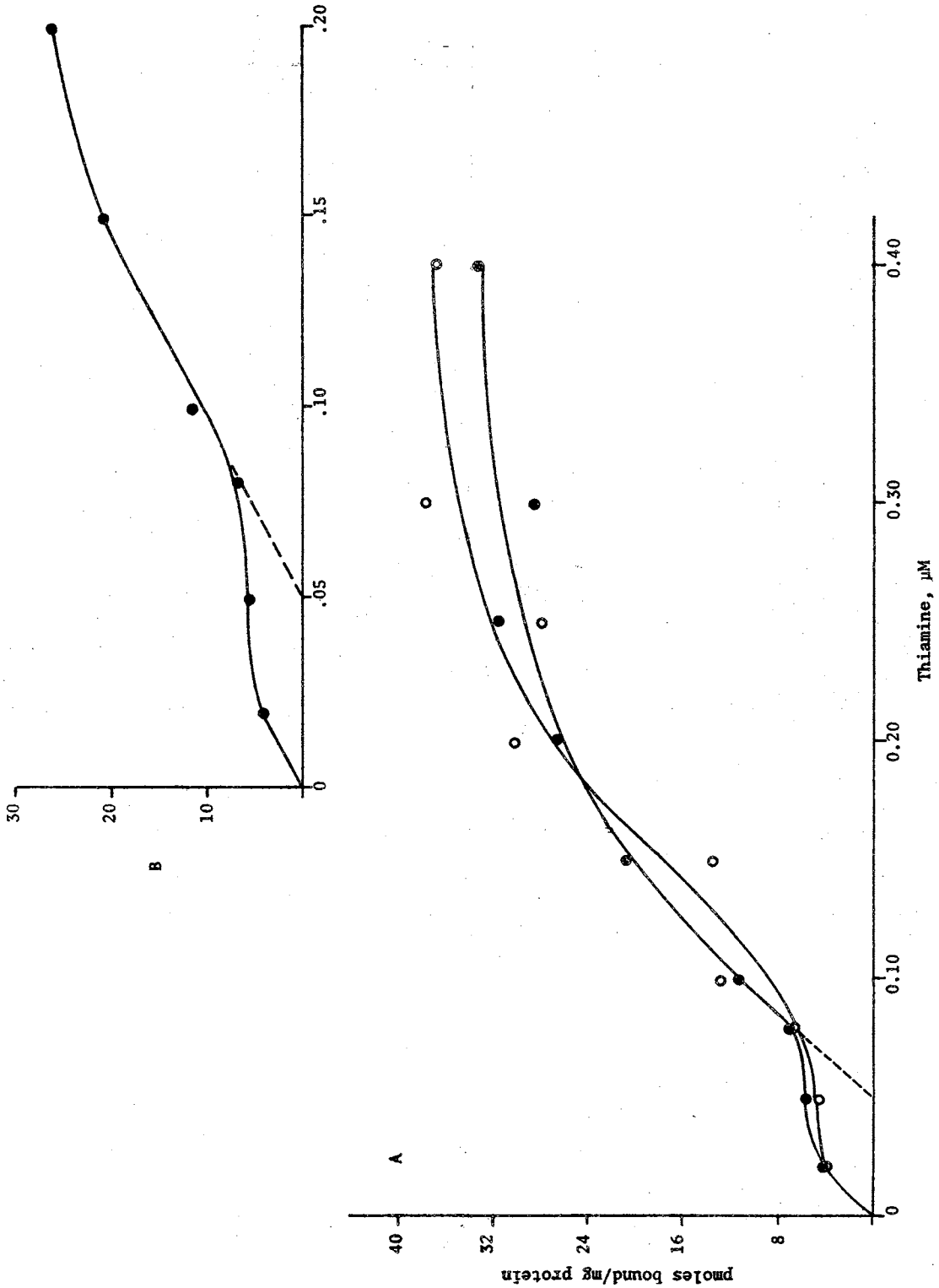
Effect of Thiamine Concentration and Energy Source on Steady State Levels of Thiamine

Equilibrium dialysis of the membranes from shocked cells with thiamine concentrations from 0.02 to 0.50 μM in the presence and absence of glucose gave the results shown in Figure 15. A double saturation was observed with all membrane preparations from shocked cells, though the estimated K_d's varied from 1 to 6 x 10^{-8} M for the first saturation and 1.2 to 2.8 x 10^{-7} M for the second. Relative amounts of thiamine bound at each saturation varied widely. Both the K_d's and amounts bound depended upon a number of parameters. A discussion of these parameters and their effects upon the two K_d's and saturation levels will be undertaken in the remainder of this chapter.

The second saturation does not extrapolate through the origin, but

Figure 15. Effect of Thiamine Concentration and Energy Source on Equilibrium Thiamine Levels

Membranes prepared from shocked cells were assayed for thiamine binding activity at a membrane protein concentration of 0.60 mg/ml in 0.5 M K-PO_4, pH 6.6. The standard assay mixture was used with (●) and without (O) glucose. Equilibrium dialysis was performed for 16 hr at 25°. Insert shows expansion of the lower thiamine concentration range.


A
B
pmoles bound/mg protein
Thiamine, μM
0
8
16
24
32
40
0.10
0.20
0.30
0.40
10
20
30
.05
.10
.15
.20

rather through about 5 x 10^{-8} M (insert). This observation precludes, or at least minimizes the possibility of, the existence of two separate entities or sites at the membrane surface which are equally accessible for binding of thiamine but which differ in affinity for ligand. At least two possibilities, which may be experimentally difficult to distinguish among in the complex membrane system, remain open for exploration. (1) This phenomenon might be explained by the presence on the membrane surface of one protein with two binding sites, one of which does not become accessible until the other is saturated. Saturation of the first site might result in a conformational change in the protein, exposing the second site for binding. (2) The two activities may represent two distinct proteins involved in separate transport systems, one for the transport of lower and the other for higher concentrations of exogenous thiamine. Many of the experiments to be discussed were designed to elucidate the basis for the biphasic accumulation phenomenon.

Figure 15 also indicates that the thiamine uptake in shocked cell membranes is not dependent upon metabolic energy, since the binding curves for the membranes with and without glucose were nearly identical. K_d's for the two saturations are 1.2 x 10^{-8} M and 1.6 x 10^{-7} M, respectively, and these K_d's are affected little if at all by the presence of glucose. The levels of thiamine accumulated at the two saturations are 7 and 34 pmoles/mg. Based on the gross estimation of 100,000 as the number of binding sites on a whole cell membrane, assuming one membrane vesicle/cell (85), this amount of thiamine associated with the membrane may reasonably be explained on the basis of binding alone. This explanation is compatible with the observation that there is no effect on accumulation by energy source, as is seen in the case of whole cell

membranes. The binding process alone would not be expected to be energy-dependent. These results suggest that two components (or sites) for thiamine transport may have become uncoupled from their energy production systems as a result of the stress placed on the membrane during osmotic shocking and membrane preparation procedures. Alternatively, shocked cell membranes may have lost components of the energy production pathway. The results of these two alterantives would be the same, i.e., loss of energization to the transport system. This hypothesis is substantiated by the drastic (200-1000 fold) decreases in accumulation by membranes from shocked cells as compared to membranes from intact cells in the presence of exogenous energy source. A number of diverse studies support the hypothesis of energy production uncoupling from transport as the basis for the apparent energy independence and binding alone as the basis for the remaining association of thiamine with the membrane. These are summarized in Table VI.

Thiamine levels found in whole cell membranes in the presence of several energy inhibitors, a chelating agent, a detergent and a compound which obliterates transport by disruption of the membranes are comparable to those found at the two saturations in the shocked cell membranes, with or without energy source. These results suggest that uncoupling of transport from energy production gives essentially the same results as chaotropic agents such as detergent and phenethyl alcohol, i.e., obliteration of transport. EDTA may also be considered a chaotropic agent, since divalent cations, particularly Mg^{2+}, are necessary for membrane integrity. Since all of these transport disrupters give comparable remaining levels of thiamine, these levels probably represent binding only.

TABLE VI

STUDIES WHICH SUBSTANTIATE ENERGY-TRANSPORT UNCOUPLING AND BINDING HYPOTHESES

	Data Obtained From Figure or Table	$Thiamine_{max_1}$ pmoles/mg	$Thiamine_{max_2}$ pmoles/mg
Shocked Cell Membranes			
+ Glucose	15	7	34
- Glucose		7	34
Whole Cell Membranes			
+ Glucose	8		890
+ 10 mM Azide	9	10	46
+ 10 mM EDTA	II	5	23
Ca^{2+} Replacing Mg^{2+}	10	10	36
+ F^-	II	3	20
+ CN^-	II		26
+ 0.1% Triton		6	10
+ 10 mM Phenethyl Alcohol		7	23

Experiments were performed as previously described for both shocked cell and whole cell membranes. Phenethyl alcohol was added to standard equilibrium dialysis mixture after addition of thiamine.

Mg^{2+} Effects on Equilibrium Thiamine Levels

The role of Mg^{2+} in thiamine transport involves specific effects as well as general membrane effects. In whole cell membranes omission of exogenous Mg^{2+} from membranes containing enough Mg^{2+} to retain their

barrier functions sharply decreases the amount of thiamine which can be accumulated. Table VII shows the effects of witholding exogenous Mg^{2+} from shocked cell membranes.

TABLE VII

EFFECTS OF Mg^{2+} ON EQUILIBRIUM THIAMINE LEVELS

	K_{d_1}, M x 10^8	K_{d_2}, M x 10^7	Maximum Thiamine Levels, pmoles/mg	
			Saturation 1	Saturation 2
No Mg^{2+}	6.5	2.7	15	55
1 mM Mg^{2+}	1.4	2.3	5	30

These experiments were performed at a [membrane protein] of 0.60 mg/ml according to the standard equilibrium dialysis method, except that in one set Mg^{2+} was omitted.

Absence of added Mg^{2+} increases both the K_d's and maximum thiamine levels at both saturations. K_{d_1} is increased 5-fold and K_{d_2} 1.5-fold, while the maximum thiamine levels at saturation 1 is increased 3-fold and at saturation 2 almost 2-fold. Assuming that the thiamine association with shocked cell membranes is binding only, the decreased K_d's in the presence of Mg^{2+} indicate a role for Mg^{2+} in enhancing affinity for both sites. The lower thiamine levels effected by Mg^{2+} suggest a possible Mg^{2+}-mediated release of thiamine from the transport component. Since accumulation in whole cell membranes is enhanced by Mg^{2+}, the release of thiamine by Mg^{2+} on the inner side of the membrane, thereby facilitating

the accumulation process, would explain the apparently discrepant effects of Mg^{2+} on thiamine uptake between whole and shocked cell membranes. These results suggest that there may be gross differences between the two and that the shocked cell membranes probably do not retain their barrier function.

Competition of TPP With Thiamine

The effect of thiamine pyrophosphate on equilibrium thiamine levels at thiamine concentrations representing the two saturations is shown in Table VIII. At the first saturation concentration there is a 37% inhibition at a 100-fold excess of TPP to thiamine. The estimated $I_{(0.5)}$ at this thiamine concentration is 1.6×10^{-6} M. At the second concentration there is no inhibition at TPP:thiamine ratios up to 100. These results suggest that the first saturation represents an accessible component, whereas, the second represents a carrier molecule within the membrane whose thiamine binding site is not accessible to the surface and therefore not inhibitable by the competitor. Increasing TPP concentration effects exposure of the second component (or site), analogous to the effect of higher thiamine concentration, such that greater amounts of thiamine are bound at saturation 2 in the presence than in the absence of TPP.

TABLE VIII

COMPETITION BY TPP

TPP/Thiamine	Thiamine Bound, pmoles/mg	
	0.05 μM Thiamine	0.20 μM Thiamine
0	12.1	28.6
10	10.4	38.7
100	7.6	34.3
$I_{(0.5)}$	1.6×10^{-6} M	----

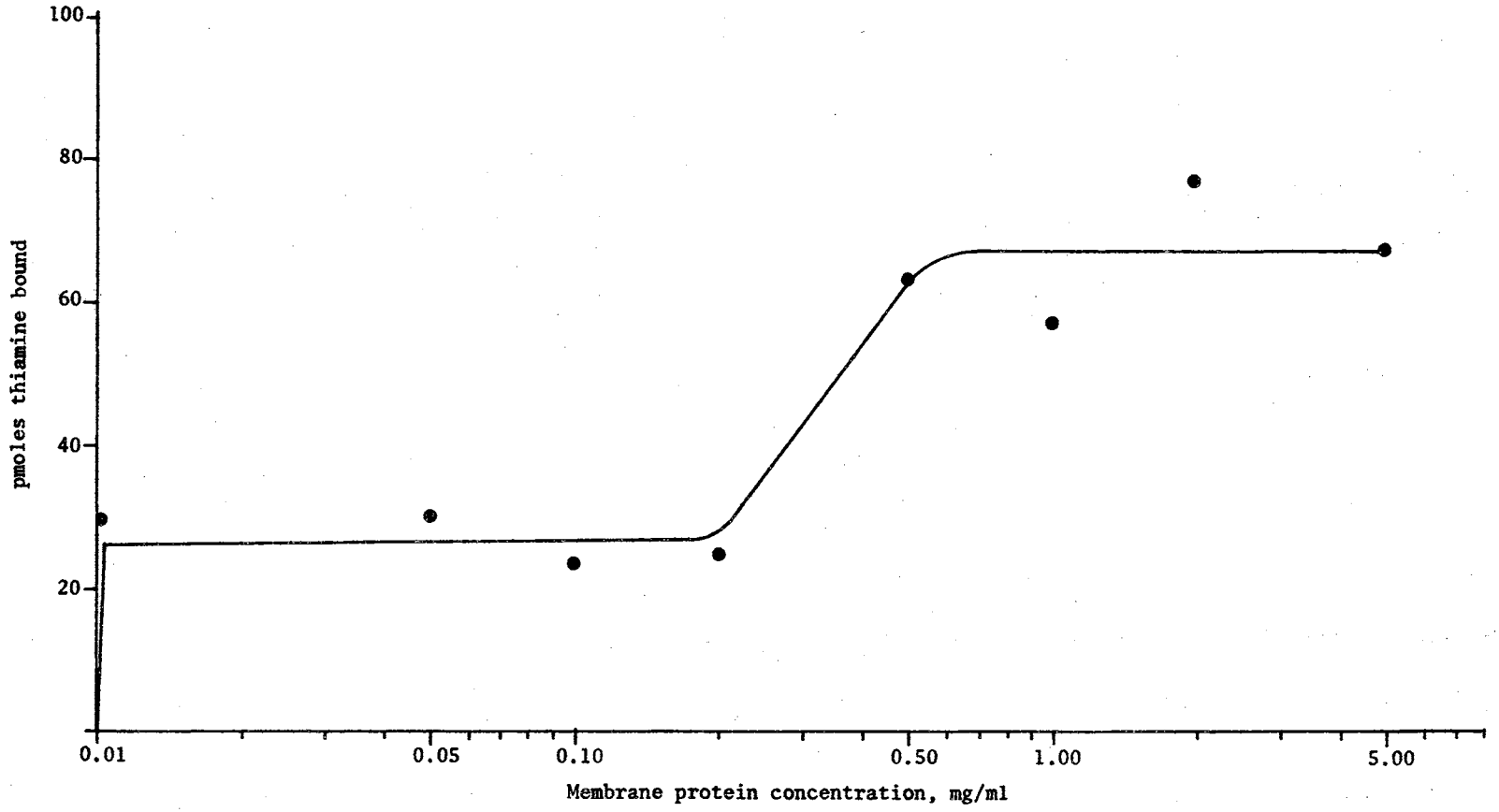
Equilibrium dialysis was performed under standard conditions.

Effect of Shocked Cell Membrane Concentration on Equilibrium Thiamine Levels

Thiamine accumulation in shocked cell membranes is not a linear function of concentration of membrane protein, as shown in Figure 16. These results differ greatly from those obtained with whole cell membranes, in which a linear relationship between accumulation and concentration exists. The complex behavior exhibited by these membranes with respect to variation of membrane concentration substantiates the suggestion that membranes prepared from shocked cells differ significantly from the membrane vesicles prepared from whole cells. Surface alterations which allow greater inter-membrane interactions in the shocked cell membranes might explain the anomalous behavior of these membranes on variation of concentration. Aggregation effects between surfaces could mask binding sites such that they are inaccessible to ligand. Further increase in membrane concentration might allow some of the fragmented

Figure 16. Effect of Membrane Concentration on Equilibrium Thiamine Levels

Equilibrium dialysis was performed under standard conditions using a thiamine concentration of 0.5 μM and a membrane protein concentration range of 5 μg/ml to 5 mg/ml.


100
80
60
40
20
pmoles thiamine bound
0.01
0.05
0.10
0.50
1.00
5.00
Membrane protein concentration, mg/ml

membranes to interact in such a manner that on reaching a critical membrane concentration, they reform vesicles. At this point uptake into the vesicles could account for the second stage of the biphasic phenomenon.

Fractionation of a Shocked Cell

Membrane Preparation

That the shocked cell membranes are fragile and easily disrupted is shown in Table IX. A preparation of shocked cell membranes made according to the standard procedure was subjected to a series of washes, resuspension and centrifugation, all of which were effective in removal of membrane material capable of binding thiamine. Freeze-thawing and washing and resuspending at high ionic strength phosphate were the most effective treatments for release of thiamine-binding material.

Table X compares the binding data on membrane preparation, supernate from centrifugation of the freeze-thawed membrane preparation and this supernate passed through an XM-50 Diaflo membrane. Assay of the supernate gave the typical biphasic curve. Both K_d's were decreased slightly, and the specific activities at the two saturations were unchanged (Table X). These results suggest that 1) smaller fragments which were not sedimented during centrifugation or 2) a single component with two binding sites accounted for the specific binding in the supernate. To differentiate between the two possibilities a freeze-thawed aliquot of this membrane preparation was centrifuged at 33,000 x g, and the supernate was passed through an XM-50 Diaflo membrane, which excludes components of molecular weight greater than 50,000. The typical biphasic binding curve was obtained for the filtrate. The K_{d_1}

TABLE IX

THIAMINE BINDING TO WASHES FROM SHOCKED CELL MEMBRANES

Wash Number	Treatment	Thiamine Bound, pmoles/mg
1	1	3.2
2	1	5.7
3	1	5.7
4	2	19.6
5	1	5.6
6	1	14.6
7	3	10.4
8	1	30.4
9	4	40.6
10	4	80.4

A single sample was sequentially treated as indicated. Binding assays were performed by equilibrium dialysis according to the standard procedure.

1: Washed in 0.1 M K-PO_4, pH 6.6, containing 10 mM EDTA and centrifuged at 16,000 x g for 30 min. at 4°.

2: Stored overnight in 0.5 M K-PO_4, pH 6.6, at -15° and centrifuged as above.

3: Washed in 0.1 M K-PO_4, pH 6.6, and centrifuged.

4: Washed in 0.5 M K-PO_4, pH 6.6, and centrifuged.

TABLE X

COMPARISON OF BINDING DATA FROM MEMBRANE PREPARATION, SUPERNATE AND ULTRAFILTRATION FILTRATE

Sample	K_{d_1} x 10^8	K_{d_2} x 10^7	Specific Activity 1, pmoles/mg	Specific Activity 2, pmoles/mg
Membrane Suspension	4.5	2.7	12	53
Supernate[a]	2.4	1.6	16	53
Filtrate[b]	2.4	2.9	115	630

All assays were performed by equilibrium dialysis under standard assay conditions.

[a]The membrane preparation ([protein] = 0.60 mg/ml) was centrifuged at 16,000 x g for 30 min. at 4°. The supernate was assayed at a protein concentration of 0.164 mg/ml.

[b]Membrane suspension (0.60 mg/ml) was centrifuged at 33,000 x g for 30 min. at 4°. The supernate was passed through an XM-50 Diaflo membrane at 4°. The filtrate was assayed at a protein concentration of 0.02 mg/ml.

was the same as that found in the supernate and K_{d_2} was the same as that in the original membrane suspension. However the specific activities were increased 10-fold at saturations over those in the supernate and filtrate. As with the membrane suspension, the second saturation did not extrapolate through the origin.

The results from this experiment show that there is a component of molecular weight less than 50,000 which is solubilized from the shocked cell membrane which binds thiamine specifically and exhibits the same general binding properties as those of the membrane suspension. The

thiamine binding protein, which has a molecular weight of 40,000, is a candidate for such a component. However, purified TBP gives a normal double reciprocal plot (81). The component fractionated from the membrane supernate may be a separate transport component of one (or both) of the two postulated thiamine transport systems.

CHAPTER V

WHOLE CELLS

Studies on isolated membranes can yield valuable information on cellular structure and function. However, these studies should be correlated whenever possible with studies on the intact cell. These correlations can frequently be accomplished more easily in studying function than in studying structure, since components may be assayed by activity and need not be isolated.

Active transport is generally regarded as a complex process involving directly one or more components and indirectly a number of others, including the electron transport system. In addition multiple transport systems exist for several substrates, most notably galactose (44). Net transport flux is the difference between influx and efflux of substrate. In some systems, e.g., galactose transport (88), the entry and exit processes are mediated by separate components, and the entry and exit systems have been partially characterized with respect to the role of the galactose binding protein and energy coupling (89).

Studies on isolated membranes of E. coli Crookes described in the preceding chapter suggested that thiamine transport may not be a single system process. For this reason it was deemed necessary to look at more than just initial rates of uptake in whole cell studies. Introduction of a new parameter was routinely accompanied by a time course study before an assumption was made that initial rates would suffice to describe the

effects of that variable on thiamine uptake.

Effect of Thiamine Concentration on Uptake

That the whole cells also display a complex thiamine transport process was demonstrated by variation of curve shapes under a variety of conditions. Figure 17 illustrates an example of variation of shape of the uptake curve with a given parameter, concentration of thiamine. A thiamine concentration of 0.10 μM gave linear uptake at short times and approached a saturating level. In contrast the higher concentrations gave more irregular curves. At 0.20 and 1.0 μM thiamine immediate uptake ensued, followed by a plateau at 0.20 μM, and a decreased rate at 1.0 μM, and subsequent hyperbolic kinetics. At higher thiamine concentrations (> 0.10 μM) these irregular curves were obtained consistently in experiments carried out under a number of different conditions. All experiments performed with 0.10 μM thiamine gave the simpler uptake curve.

These results suggest that two transport systems may be operative at higher thiamine concentrations. This has been shown to occur with histidine transport in S. typhimurium (7). Substantiation for this hypothesis is provided by the Lineweaver-Burk plot shown in Figure 18, depicting the effect of thiamine concentration on initial velocity of uptake. In both Tris and phosphate buffers at pH 8.0 whole cells show two processes, a sharp break occurring at concentrations less than 0.20 μM in both. The apparent K_m and the V_{max} for the high concentration process were approximately the same in both buffers. The slight substrate inhibition effect noted in the presence of phosphate was seen routinely with this buffer and occasionally with Tris.

The results of thiamine concentration studies on whole cells

Figure 17. Thiamine Uptake at Three Concentrations of Thiamine

Uptake studies were performed at 0.10 (O), 0.20 (▲) and 1.0 (●) μM thiamine under standard conditions on cells suspended in 10 mM Tris, pH 8.0, to a concentration giving an A_{560} of approximately 0.10. Sampling and counting were performed in the usual manner, and results were plotted as pmoles/mg dry cell weight vs. time.

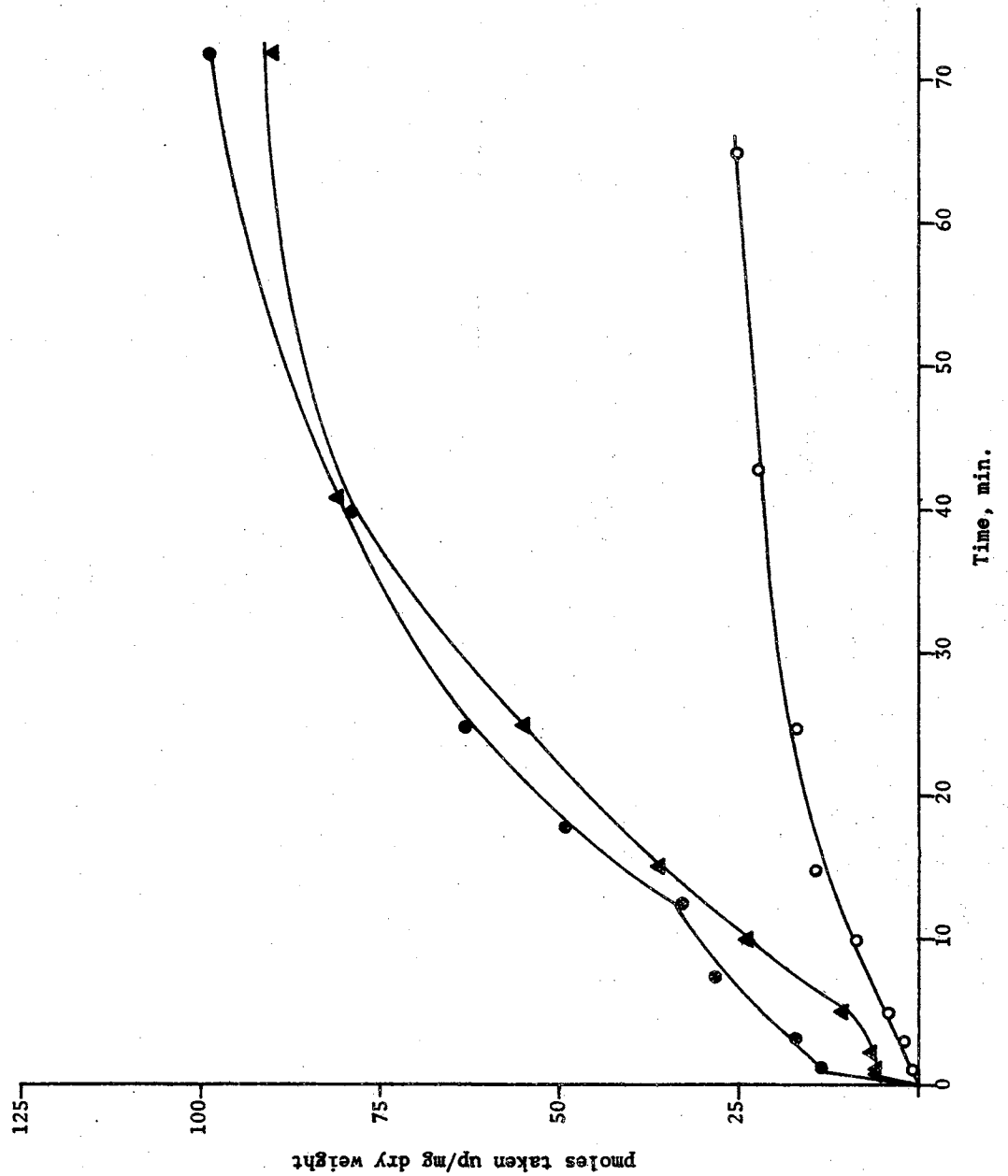
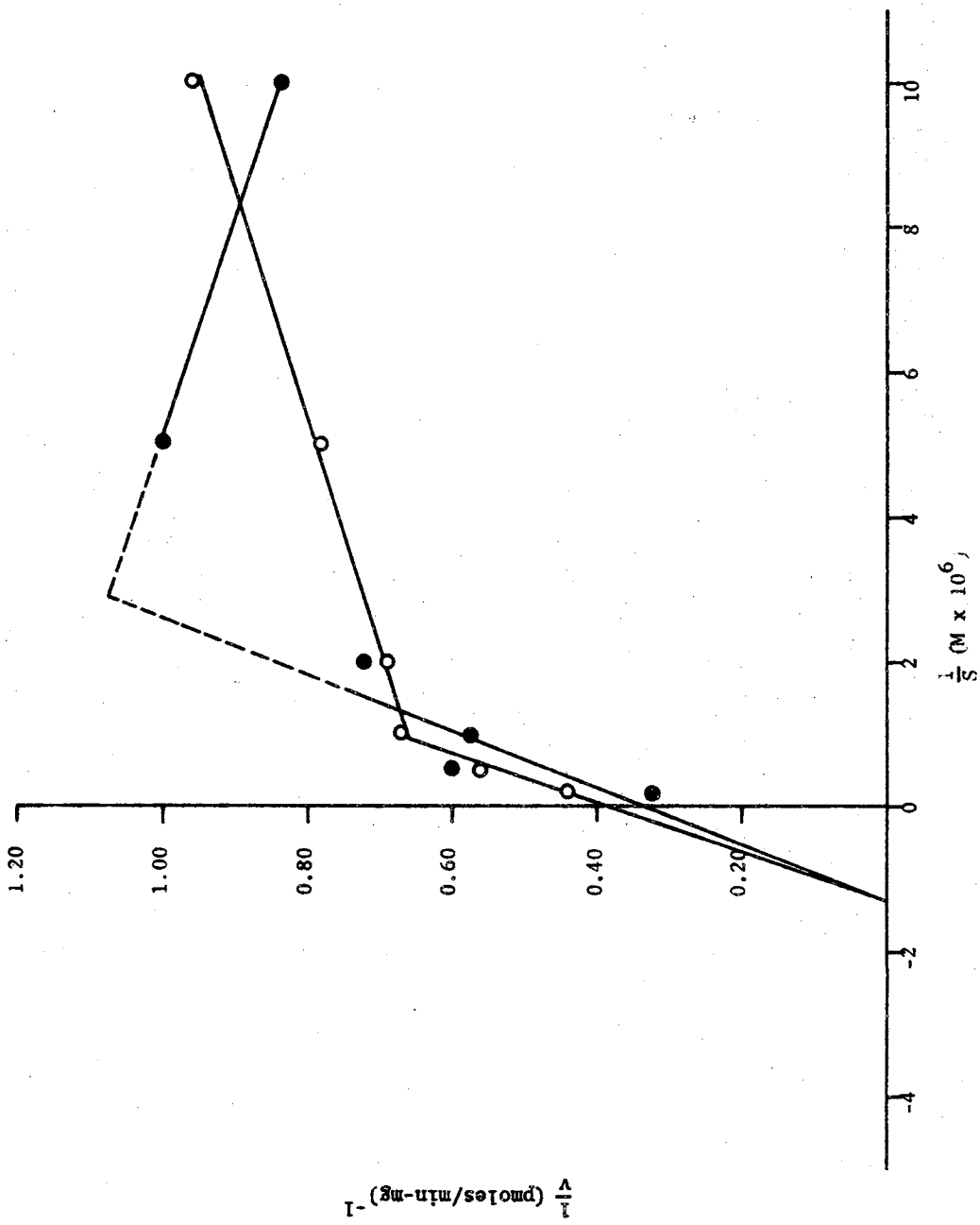

125
100
75
50
25
0
10
20
30
40
50
60
70
Time, min.
pmoles taken up/mg dry weight

Figure 18. Effect of Thiamine Concentration on Uptake at 10 Minutes

Two aliquots of a mid-log M-9-grown culture were collected by Millipore filtration. One was washed with 0.1 M K-PO_4, pH 8.0, and the other with 0.1 M Tris, pH 8.0. The washed cells were suspended in the appropriate buffer, and uptake studies were done by the standard procedure. The mixtures were sampled at 5 and 10 minutes, and the 10 minute values were plotted according to the Lineweaver-Burk method. ● = phosphate, O = Tris.


1.20
1.00
0.80
0.60
0.40
0.20
0
-2
-4
2
4
6
8
10
$\frac{1}{S}$ (M x 10^6)
$\frac{1}{v}$ (pmoles/min-mg)$^{-1}$

correlate well with those obtained with isolated membrane preparations. The Lineweaver-Burk plots for both show a sharp break around 0.10 μM thiamine, and the substrate inhibition effect at lower concentrations is demonstrated by both. However, higher concentrations of thiamine in the isolated membrane produced a substrate activation effect such that constants could not be obtained. This observation suggests differences between the membrane surfaces of whole cells and isolated membranes such that caution must be exercised in extrapolating results obtained with membranes to the situation which exists in whole cells.

Effect of Energy Source on Uptake

Figure 19 shows the effect of glucose addition to cells incubated at 37° in M-9 medium. An immediate saturating accumulation occurs in the absence of exogenous energy source. On addition of glucose an immediate increase in uptake occurs, followed by a decrease, then an increase to the level attained prior to the drop. This level is maintained as long as an hour after glucose addition.

These results suggest that an immediate uptake occurs in the absence of glucose, but this process ceases very quickly, presumably due to consumption of all available endogenous energy source. The effect seen on addition of glucose indicates not only that thiamine uptake is an energy-requiring process, but also that energy may be required for an exit process. The same results are seen in thiamine exit studies in the presence of glucose (Figure 20). Resuspension of thiamine-loaded cells in medium containing glucose causes an immediate loss of thiamine followed by re-entry to the initial level.

Table XI indicates the greater effectiveness of glucose than other

Figure 19. Effect of Glucose on Uptake

Late log cells were washed once with M-9 medium without glucose and Mg^{2+} and suspended in M-9 medium containing 10^{-4} M EDTA. Uptake was followed according to the standard procedure at 37° for 50 minutes, at which time glucose was added to 10 mM and the assay continued. Sampling and counting were done in the usual manner. The results are plotted as pmoles taken up vs. time.

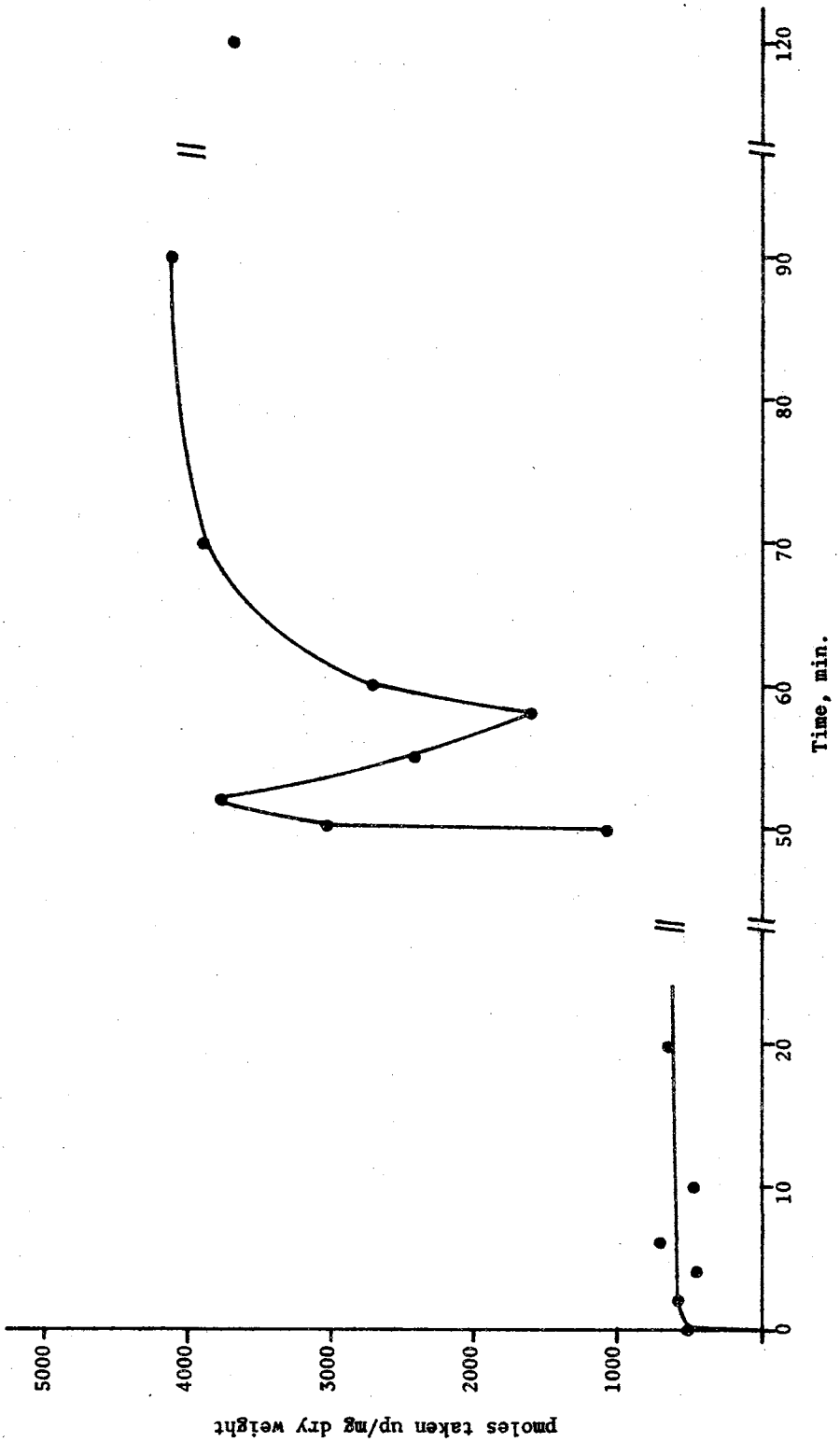
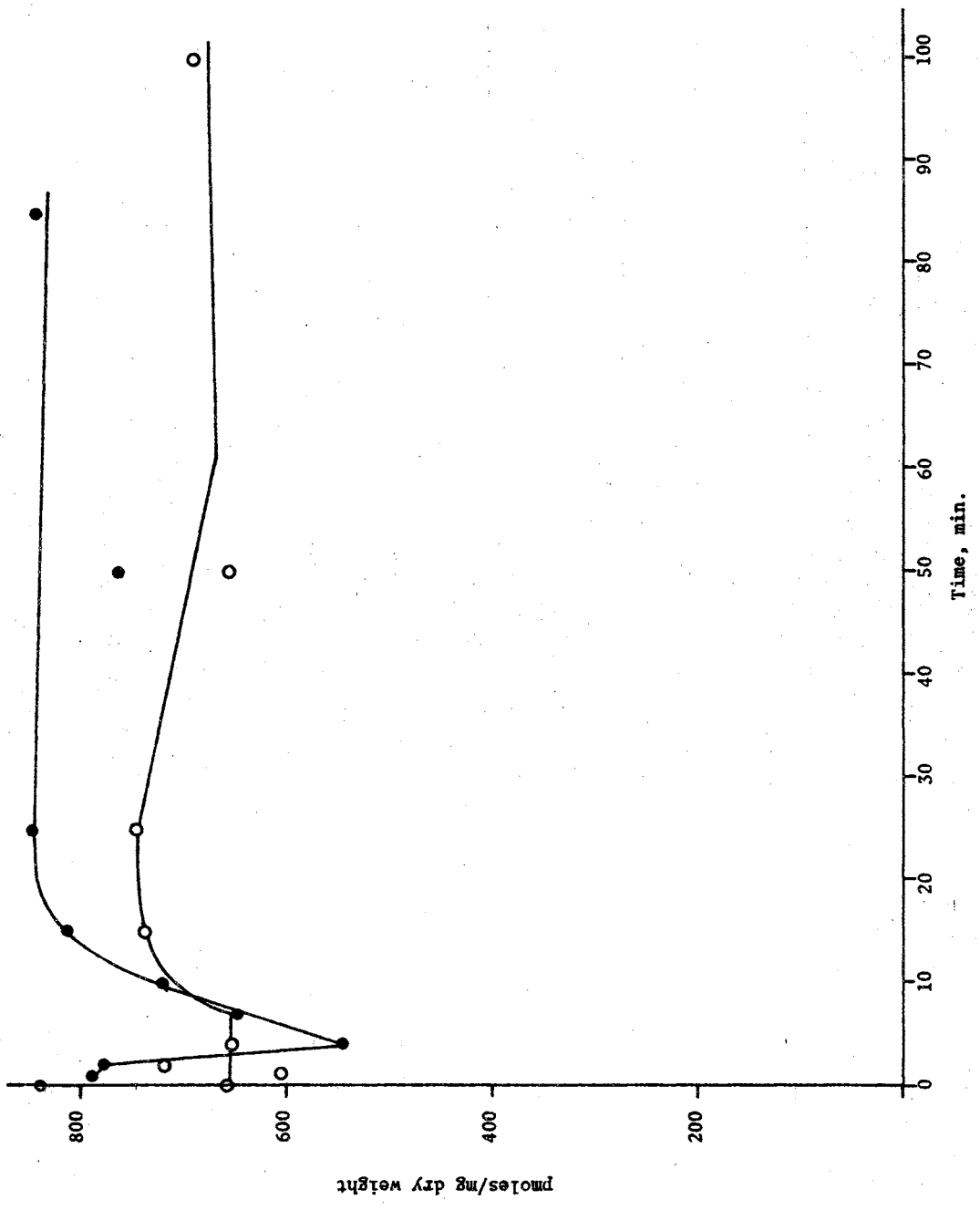

pmoles taken up/mg dry weight
5000
4000
3000
2000
1000
0
10
20
50
60
70
80
90
120
Time, min.

Figure 20. Effect of Glucose on Exit

A log phase culture of cells was harvested by Millipore filtration and washed with M-9 salts containing no glucose. The cells were resuspended in the same buffer containing 0.2 mg/ml chloramphenicol and 1 μM ^{35}S-thiamine. After incubation with shaking at 37° for 2 hours, the suspension was centrifuged in two aliquots at 16,000 x g for 15 minutes at room temperature. One was suspended in M-9 without glucose (O) and the other in M-9 containing 10 mM glucose (●). Immediately after resuspension aliquots were taken to monitor exit in the same manner as for uptake studies. Results are plotted as pmoles thiamine associated with the cells vs. time.


pmoles/mg dry weight
800
600
400
200
0
10
20
30
40
50
60
70
80
90
100
Time, min.

energy sources in energizing thiamine transport. Initial velocities in the presence of D,L-lactate at pH 6.6 and 8.0 are, respectively, 70% and 79% as fast as with glucose, and D,L-lactate energizes accumulation of ∿ 48% and 66%, respectively, of the glucose level at 70 minutes. These results indicate that glucose is energizing a process other than or in addition to that involving lactate, which is presumed to be the D-lactate dehydrogenase system. Uptake in 10 mM pyruvate is 60% as fast as in glucose, and accumulation is 70% that of the glucose-energized level at 70 minutes. The magnitude of inhibition of uptake in 100 mM pyruvate may suggest a role for this compound, the product of both pyruvate kinase and D-lactate dehydrogenase activity, in controlling transport by these mechanisms. The results shown in Table XII suggest that lactate too may be serving as an energy source for a different transport system. Oxamate, a specific inhibitor of D-lactate dehydrogenase, reduces the initial velocity of uptake at 1 μM thiamine by 88% and the maximal accumulation by 92%.

Keeping the cells in a reduced state with mercaptoethanol reduces the initial velocity by ∿ 50%, while affecting the amount accumulated but slightly (12%). Since the D-lactate dehydrogenase-linked transport system requires both oxidation and reduction of sites to function, this mechanism is probably hindered by a reducing environment. The PEP-phosphotransferase system involves a critical -SH group, that at the active site of Enzyme I, for activity; therefore this system might be stimulated by a reducing environment. The lack of net effect of mercaptoethanol on accumulation may be explained on this basis.

To rule out the possibility that metabolism of the mercaptoethanol by the cell accounts for the lack of effect after a period of time, cells

TABLE XI

EFFECTIVENESS OF LACTATE AND PYRUVATE IN ENERGIZING TRANSPORT

Energy Source	pH	V_i pmoles/min / mg dry weight	Thiamine Accumulated at 70 Min. pmoles / mg dry weight
Glucose, 10 mM	6.6	144	1538
D,L-Lactate, 10 mM		99	750
Pyruvate, 100 mM		---	75
Glucose, 10 mM	8.0	124	1888
D,L-Lactate, 20 mM		97	2147
Pyruvate, 10 mM		72	1344

Aliquots of M-9 cultures grown to mid-log phase were harvested by Millipore filtration, washed with 50 mM potassium phosphate buffer at pH 6.6 and 8.0 and resuspended in the appropriate buffer. Uptake studies were performed according to the standard procedure.

TABLE XII

INHIBITION OF UPTAKE BY OXAMATE AND -SH

	V_i pmoles/min / mg dry weight	Thiamine Accumulated at 70 Min. pmoles / mg dry weight
Control	61.1	1506
10 mM Mercaptoethanol	35.5	1306
10 mM Oxamate	7.8	128

A log phase M-9 culture of cells was harvested by Millipore filtration, washed with 50 mM K-PO_4, pH 6.6, and suspended in the same buffer. All suspensions were incubated for 20 minutes at 37° prior to initiation of uptake by addition of ^{35}S-thiamine.

were treated with N-ethylmaleimide prior to initiation of uptake. Table XIII shows that the sulfhydryl reagent reduces the initial velocity by 23% and maximal accumulation by 12%. These results indicate that thiamine transport is not completely dependent upon -SH-containing species or that not all of the critical sulfhydryls involved in thiamine transport are accessible for reaction with NEM.

TABLE XIII

EFFECT OF N-ETHYLMALEIMIDE ON UPTAKE

	v_i pmoles/min / mg dry weight	Thiamine Accumulated at 70 Min. pmoles / mg dry weight
Control	61.4	1015
NEM-Treated	47.0	875

A log phase culture of cells was harvested by Millipore filtration, washed with 50 mM Tris, pH 7.2, and resuspended in the same buffer containing 0.2 mg/ml chloramphenicol. The suspension was divided into two portions. To one was added N-ethylmaleimide to 0.5 mM. The control and the reaction mixture were incubated with shaking at 25° for 1 hr. Aliquots of control and reaction mixture were filtered on Millipore filters, washed with 10 ml of buffer and resuspended in standard uptake medium. Uptake studies were done by the standard procedure.

Table XIV compares the relative effectiveness of PEP and glucose in energizing thiamine transport at two thiamine concentrations. At 0.10 μM thiamine PEP is more than 90% as effective as glucose in stimulating initial velocity and maximal uptake. Addition of fluoride greatly decreases both parameters. A more pronounced difference between energy

TABLE XIV

EFFECTIVENESS OF PEP IN ENERGIZING THIAMINE TRANSPORT

Energy Source	V_i pmoles/min / mg dry weight	Thiamine Accumulated at 90 Min. pmoles / mg dry weight
0.10 μM Thiamine		
Glucose, 10 mM	193	451
PEP, 10 mM	172	423
Glucose, 10 mM + 10 mM F^-	36	74
1.0 μM Thiamine		
Glucose, 10 mM	221	1348
PEP, 10 mM	197	520
Glucose, 10 mM + 10 mM F^-	274	669

Fluoride was incubated with the cells for 15 min. in 50 mM Tris, pH 8.0, containing Mg^{2+} and chloramphenicol prior to addition of glucose. Uptake was performed by the standard procedure.

sources is observed at 1.0 μM thiamine. The initial velocity in the presence of glucose is ∿ 20% higher than in the presence of PEP, and maximal accumulation is 2.6 times as great. Addition of fluoride actually enhances initial velocity over that in the presence of glucose alone, yet maximal accumulation is approximately half. These results indicate that at lower thiamine concentrations PEP is capable of stimulating transport to almost the same extent as glucose. In the presence of the enolase inhibitor fluoride cells are capable of accumulating thiamine by an alternate mechanism. However, accumulation is only 16% of that in the presence of glucose alone. At the higher thiamine concentration the alternate mechanism accounts for half of the thiamine uptake capability of the cell. The 24% stimulation of initial velocity in the presence of F^- may be a result of decreased pyruvate concentration in the absence of the PEP to pyruvate conversion on inhibiting enolase. The decreased pyruvate pool would then exert less of an inhibitory action on lactic dehydrogenase, whose product is also pyruvate.

Effect of Inhibitors on Uptake

Table XV illustrates the effects of inhibitors on thiamine uptake. The uncoupler DNP is the most effective, inhibiting maximal accumulation by 76%. However, quite different results are seen with the other uncoupler azide, which decreases the initial velocity by 42%, but which stimulates maximal thiamine accumulation over control levels. These results are not as disparate as they appear, however, when one considers the following facts: 1) DNP exerts effects other than on uncoupling of oxidative phosphorylation and these membrane-directed effects might play a major role in disruption of a membrane activity; 2) transport can

occur under conditions where oxidative phosphorylation is uncoupled from electron transport (1,91), and can occur in mutants which are uncoupled for oxidative phosphorylation (92); 3) azide forms a complex with any type of non-heme metal-containing enzymes (93). It may interact with a Mg^{2+}-requiring transport component to decrease the initial velocity. The stimulation of uptake at later times can be explained by the fact that uncoupling, which decreases ATP levels in the cell, stimulates the production of PEP, another major high energy phosphate compound, thereby stimulating transport by the PEP-PTS (29).

TABLE XV

EFFECT OF INHIBITORS ON UPTAKE

Inhibitor[1]	v_i pmoles/min / mg dry weight	Thiamine Accumulated at 70 Min. pmoles/mg
None	57	812
F^-	45	538
CN^-	42	510
N_3^-	33	900
DNP	--	194

[1]All inhibitors were used at a concentration of 10 mM. Uptake suspensions were incubated for 20 min. with inhibitors before addition of glucose and ^{35}S-thiamine.

Respiration is required for part but not all of the thiamine transport capability, since there is a 26% reduction in initial velocity and a 41% decrease in maximal accumulation by cyanide. The effect of fluoride, which rather specifically inhibits enolase, is quantitatively very similar to that of cyanide on both initial velocity and maximal uptake. These inhibitors may both act on a PEP-PTS-mediated portion of the thiamine transport capability of the cell.

Cyanide and fluoride have qualitatively the same effects on transport in isolated membranes and whole cells, i.e., they reduce accumulation in both. On the other hand azide and DNP show different effects on membranes and whole cells. Whereas azide stimulates accumulation in whole cells, it almost completely inhibits accumulation in isolated vesicles. DNP, which very effectively reduces thiamine accumulation in whole cells, has little effect on that activity in membrane vesicles. These results indicate differences between the membrane surfaces and/or components of whole cells and isolated membrane vesicles.

Effect of Temperature on Uptake

Thiamine transport is a temperature-dependent process, as shown in Table XVI. Initial velocity and maximal accumulation are almost doubled in going from 25 to 37°. There was essentially no association of thiamine with the cells at 4°. This observation differs from that seen at 4° with isolated membranes, which show a sizeable amount of thiamine associated with the membrane at the lower temperature. This is yet another indication of differences between the membrane surfaces of isolated membrane vesicles and whole cells.

TABLE XVI

EFFECT OF TEMPERATURE ON UPTAKE

Temperature °C	V_i pmoles/min / mg dry weight	Thiamine Accumulated at 80 Min. pmoles / mg dry weight
37	43.4	1313
25	29.9	743
4	----	0.3

Uptake was assayed by the standard procedure on late log cells in 10 mM Tris, pH 8.0, at the three temperatures.

Effect of Mg^{2+} on Uptake

Since Mg^{2+} was necessary for maximal accumulation in isolated membranes, the effect of this ion on uptake in whole cells was studied. Figure 21 shows that addition of Mg^{2+} to cells containing neither Mg^{2+} nor energy source results in an immediate 2.7-fold increase in thiamine accumulation. Subsequent glucose addition then increased accumulation another 64%. Figure 22 depicts the effect of 10 mM EDTA on uptake. These studies indicate that Mg^{2+} is necessary for maintenance of transported thiamine inside the cell.

Effect of Growth Medium on Uptake

Growth medium influences both initial velocities of thiamine uptake and thiamine accumulation capacity, as shown in Table XVII. Growth in thiamine-supplemented medium greatly reduced thiamine uptake capability.

Figure 21. Effect of Mg^{2+} on Uptake

Late log cells were washed once with M-9 medium without Mg^{2+} and glucose and suspended in M-9 medium containing 10^{-4} M EDTA. Uptake was followed by the standard procedure at 37° for 50 minutes, at which time Mg^{2+} was added to 1 mM and the assay continued. Sampling and counting were performed in the usual manner. Results are plotted as pmoles thiamine taken up per mg dry weight vs. time.

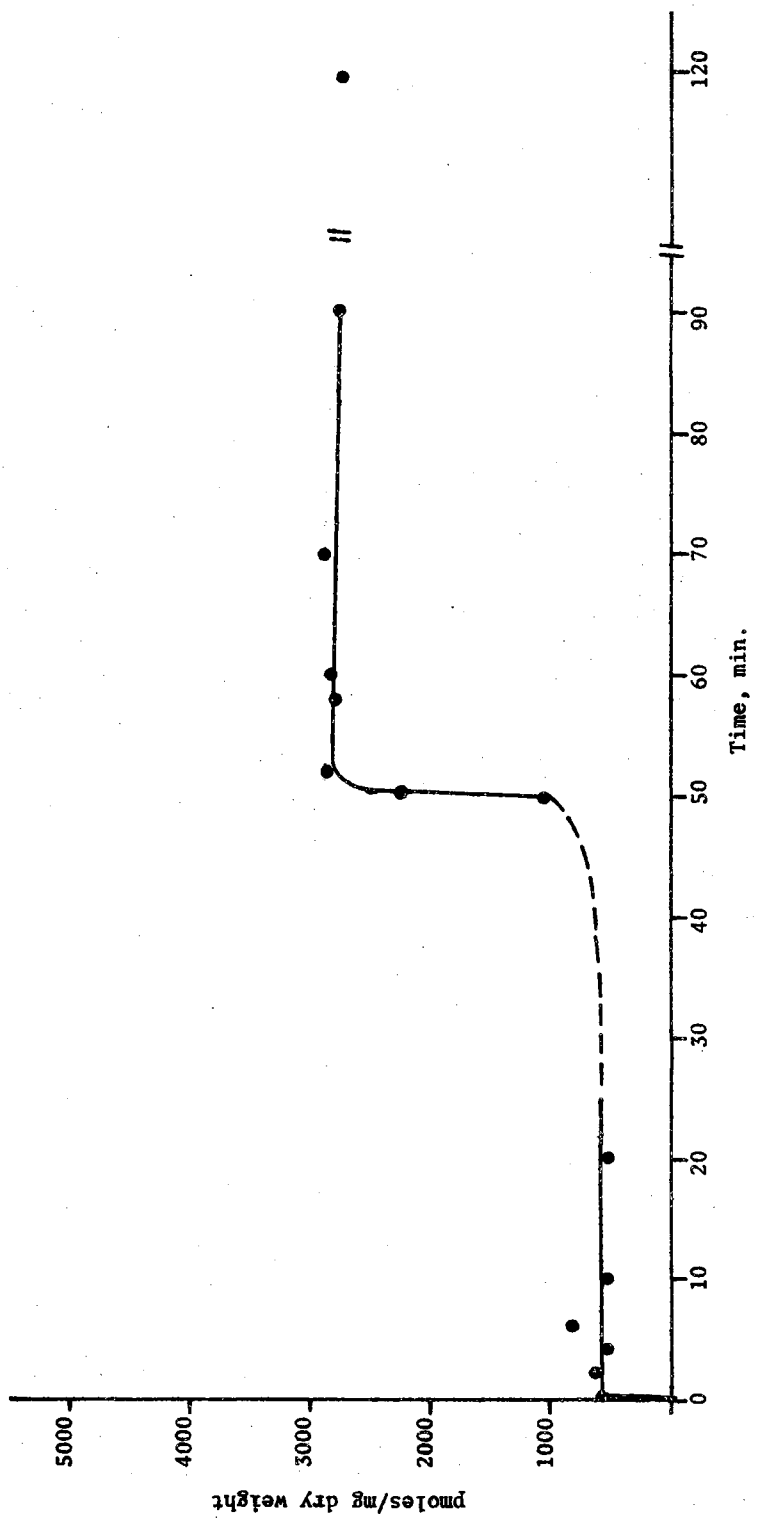

pmoles/mg dry weight
5000
4000
3000
2000
1000
0
10
20
30
40
50
60
70
80
90
120
Time, min.

Figure 22. Effect of EDTA on Uptake

A log phase culture of cells was filtered on a Millipore filter, washed with 50 mM Tris, pH 8.0, and suspended in the same buffer containing 10 mM EDTA. The suspension was incubated for 1 hour at 37°. An aliquot of the cells was filtered and suspended in the standard uptake medium and uptake followed in the usual manner. The first point was taken at 20 secs.

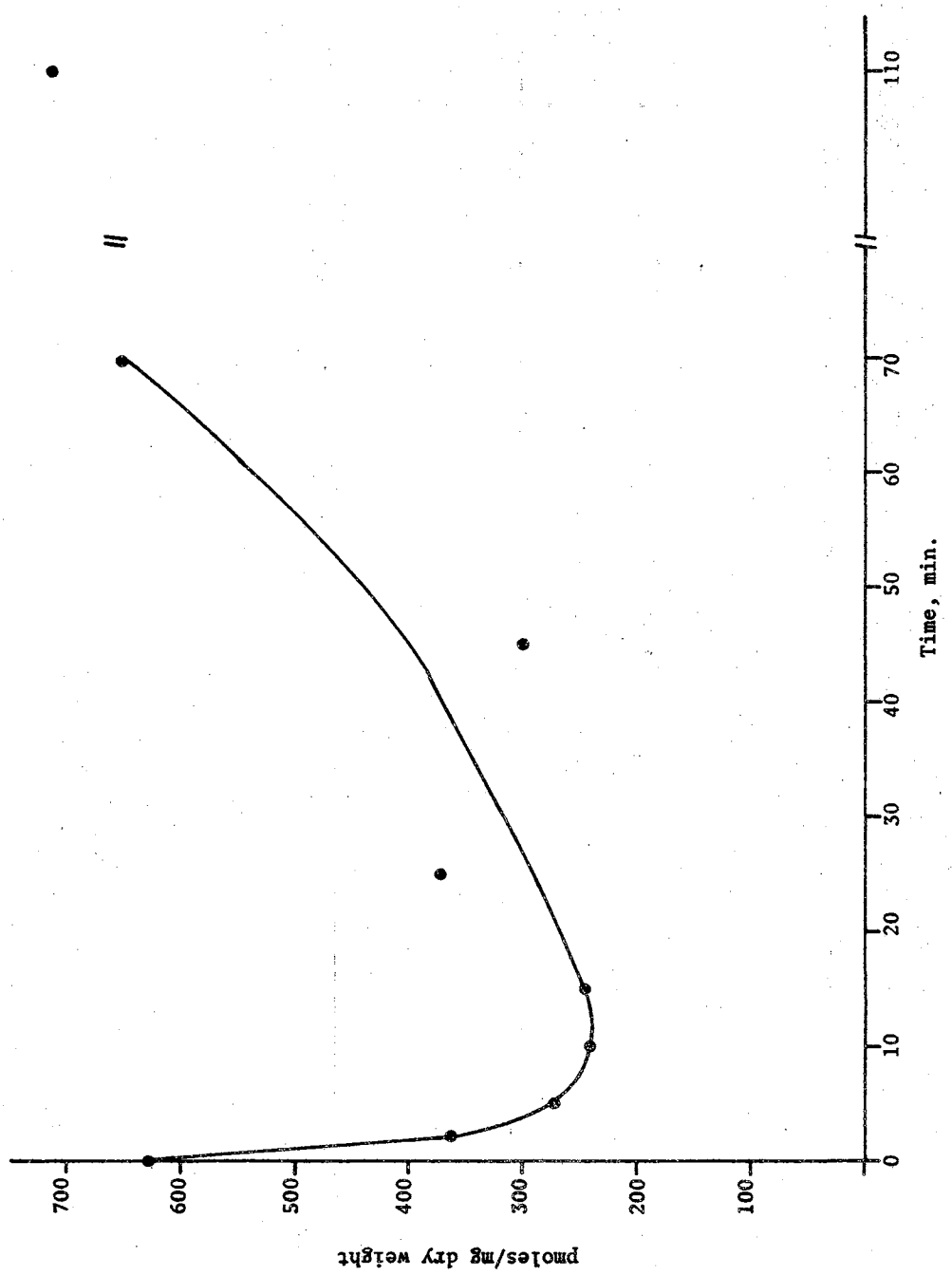

700
600
500
400
300
200
100
0
10
20
30
40
50
60
70
110
Time, min.
pmoles/mg dry weight

TABLE XVII

EFFECT OF GROWTH MEDIUM ON UPTAKE

Medium	V_i pmoles/min / mg dry weight	$Thiamine_{(70)}$, pmoles/min
Minimal Salts	40.0	1535
Thiamine, 5 mM	----	306
Adenine, 5 mM	71.4	643
Nutrient Broth	59.6	687
Low Phosphate (1 mM + 64 mM Tris)	45.5	1160
Low Phosphate + Phosphate	53.3	2828

Cells were grown to late log phase in appropriate medium. All media were M-9 salts with 0.2% glucose + the appropriate addition except nutrient broth, which was 2% tryptone. Uptake was done by the standard procedure on cells washed and suspended in 50 mM Tris, pH 8.0.

Adenine in the growth medium increases initial velocity of uptake, but decreases by 57% the capacity of the cells for thiamine accumulation compared to minimal salts-grown cells. Growth in nutrient broth also decreases the amount accumulated but does not alter initial velocity of uptake. Cells grown in low-phosphate medium given an initial velocity similar to those grown in minimal salts and accumulate almost twice as much as the adenine-or nutrient broth-grown cells. This observation was unexpected, since these cells, harvested in stationary phase, should be depleted of high energy phosphate compounds which supply energy for cellular activities. Growth in low phosphate medium must cause induction, depression or enhancement of a system which does not utilize high energy phosphate compounds for energization of thiamine transport. However, another system mediated by phosphorylation-dephosphorylation must be present also, since addition of phosphate to cells grown in low phosphate medium stimulates initial velocity and more than doubles maximal accumulation.

Effect of pH on Uptake at Various Thiamine Concentrations

Table XVIII compares the constants obtained at pH 8.0 and 6.6 in the Tris and phosphate buffers. At pH 6.6 a lower apparent K_m and higher V_{max} are obtained for the uptake experiments performed in phosphate. At this pH uptake in both buffers shows a pronounced substrate inhibition effect at thiamine concentrations less than 0.20 μM. A molecular explanation for this phenomenon might be that on increasing thiamine concentration the surface-oriented carrier for the low concentration transport system undergoes a conformational change which obscures its active site

from the surface. Then at a threshold level of substrate necessary for exposure of the higher K_m carrier the reverse occurs for this protein, i.e., a conformational change resulting in exposure of this carrier's active site takes place. Precedence for such a "threshold" phenomenon in a two-system transport process was established by Ames for the high and low affinity systems for histidine (7).

TABLE XVIII

EFFECT OF pH ON CONSTANTS OBTAINED FROM INITIAL VELOCITY STUDIES

Buffer	pH			
	6.6		8.0	
	$K_{m\ app}$ x 10^7	V_{max}, pmoles/min	$K_{m\ app}$ x 10^7	V_{max}, pmoles/min
Tris, 0.1 M	3.1	4.6	6.9	2.5
K-PO_4, 0.1 M	8.3	3.1	6.5	3.1

Uptake was followed by the standard procedure on late log phase cells which had been harvested by Millipore filtration and washed with the appropriate buffer.

As seen in Table XVIII initial rates are faster in Tris at pH 6.6 than at pH 8.0, and pH does not affect the initial rate in phosphate. However, when maximal accumulations (or initial velocities for the second phase of uptake) are compared, uptake is maximal at pH 8.0 and minimal at 6.6, as seen in Table XIX. Accumulation is higher in the presence of Tris than with phosphate, since uptake in phosphate medium tends to

saturate at 70-90 minutes, whereas no real saturation is reached in this time period with Tris.

TABLE XIX

EFFECT OF pH AND BUFFER ON MAXIMAL ACCUMULATION OF THIAMINE

Buffer	pH	Thiamine Accumulated at 70 Min. $\frac{\text{pmoles}}{\text{mg dry weight}}$
M-9 (55 mM in $PO_4^{=}$)	7.0	1979
Low Phosphate M-9 (5 mM in $PO_4^{=}$, 50 mM in Tris)	7.0	1758
K-PO_4, 50 mM	6.6	1724
K-PO_4, 50 mM	8.0	2150
Tris, 50 mM	8.0	2466

Aliquots of a late log phase culture of cells were harvested on Millipore filters, washed and suspended in the appropriate buffer. Uptake was followed by the standard procedure.

Thus the effects of pH on the two phases of the uptake curve differ, the lower pH stimulating the initial velocity and the higher pH the second phase of uptake.

Effect of Trypsin on Thiamine Uptake

One of the most direct ways to study surface localization of membrane components is to treat the membrane in some manner which will alter in a detectable fashion those components which are immediately accessible

to the medium. Chemical modification with labeled reagents is one such means; however, interpretation of results is complicated by the kinetics of reagent penetration of the membrane. Such complications are avoided by the use of high molecular weight molecules such as enzymes which will not pass through the permeability barrier without gross disruption of the membrane.

Trypsin treatment of isolated membranes afforded information difficult to obtain by other methods on accessibility of transport components to the membrane surface. The effect of trypsinization of whole cells on thiamine transport was undertaken to obtain the same type of information for the whole cell surface and to compare these results with those obtained on membrane vesicles. Figure 23 shows the effects of trypsin at 1 mg/ml under standard conditions. Under these conditions trypsin decreases the initial velocity by 75%, from 26.4 to 7.3 pmoles/min/mg dry weight. Accumulation in the trypsin-treated cells reaches a saturation earlier than in untreated cells, which continue to take up thiamine for over 2 hours. The level of thiamine in trypsin-treated cells at 72 minutes was 50% of that in untreated cells. Trypsin treatment under these conditions indicates probable accessibility of components of both transport systems to the surface.

However, milder trypsin treatment (at 30° rather than 37°) and assay at 30° gave the results seen in Figure 24. These conditions elicit opposite effects on early and late accumulation capacity. The amount of thiamine accumulated at 10 minutes in trypsin-treated cells is 22% greater than in untreated cells. At 15 minutes, however, the level in trypsin-treated cells drops to 72% of that in untreated cells and remains lower for the duration of the assay. The 70 minute level is 87% that of

Figure 23. Effects of Trypsin (1 mg/ml) Under Standard Conditions on High Activity Cells

Early log phase cells were harvested on a Millipore filter and suspended in 10 mM Tris, pH 8.0. Two aliquots were taken, one for the control (●) and the other for trypsin treatment (O) at 1 mg/ml trypsin for 1 hr at 37°. Soybean trypsin inhibitor was added to the trypsin-treated cells, and uptake was followed by the standard procedure.

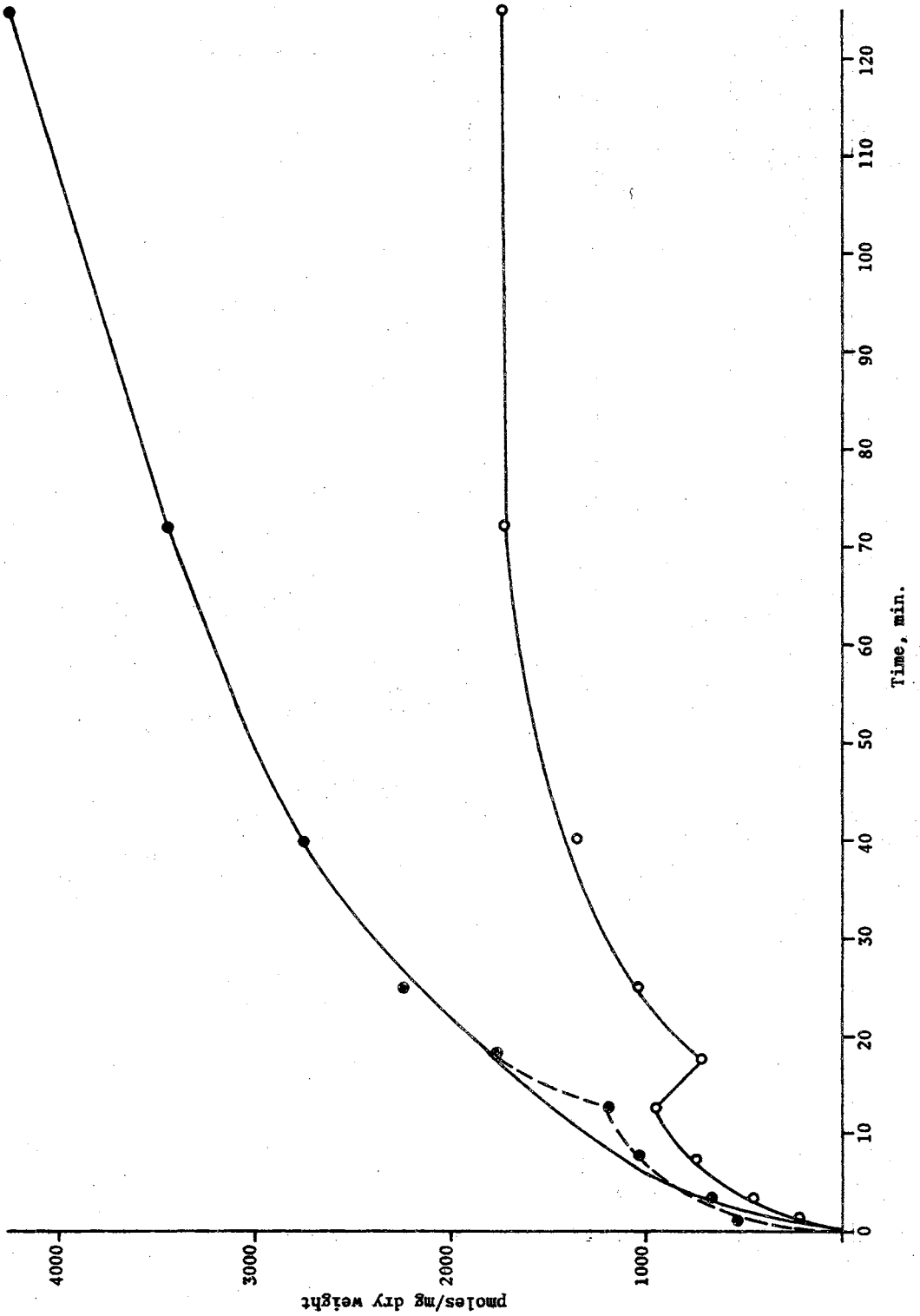
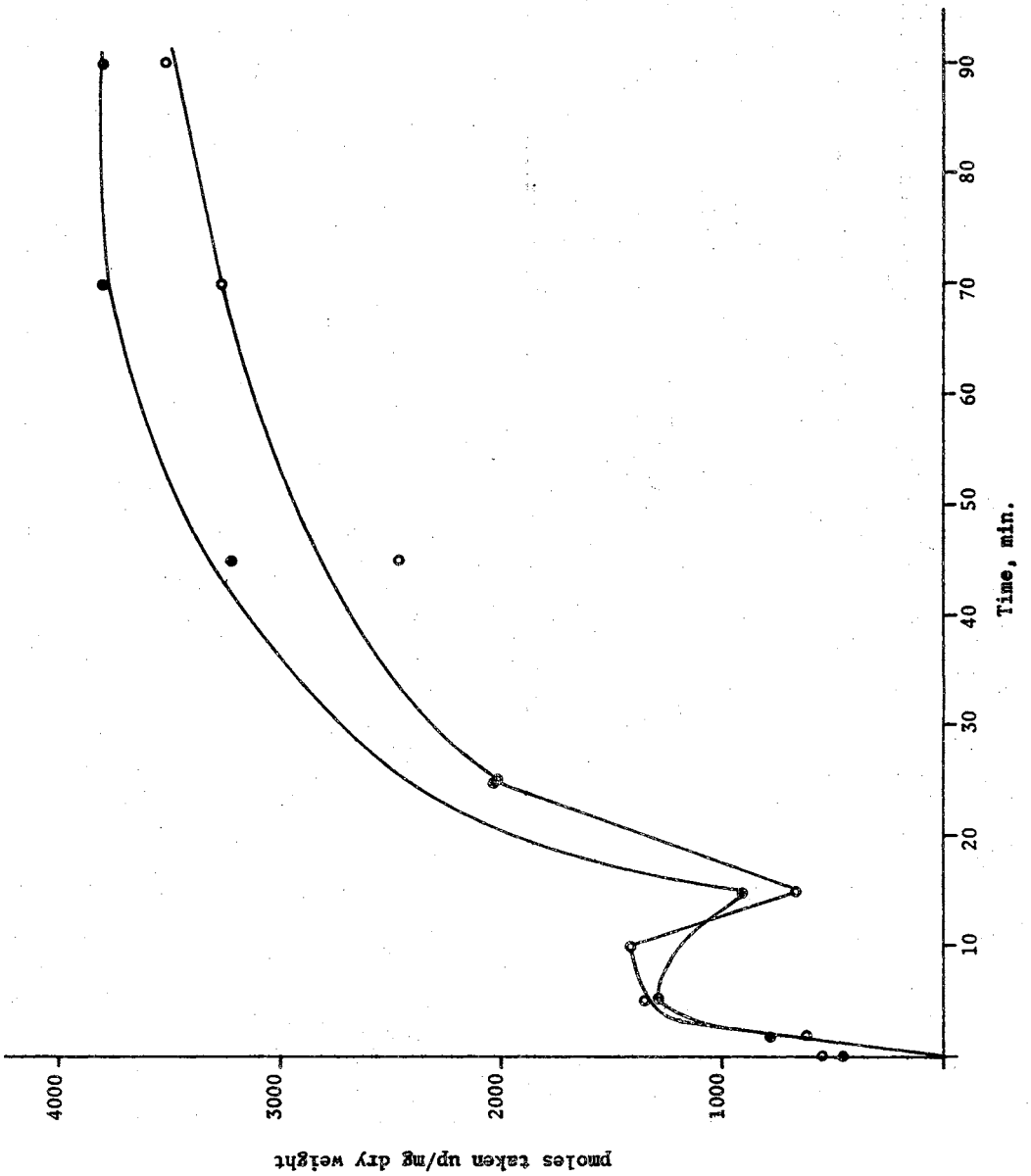

pmoles/mg dry weight
4000
3000
2000
1000
0
10
20
30
40
50
60
70
80
90
100
110
120
Time, min.

Figure 24. Milder Conditions of Trypsin Treatment: 30° for 45 Minutes

A log phase culture of cells was harvested by Millipore filtration, washed with 10 mM Tris, pH 8.0 and suspended in the same buffer. Trypsin treatment was performed at a trypsin concentration of 1 mg/ml at 30° for 45 min. The reaction was terminated and uptake performed by the standard procedures. Control (●), trypsin treatment (O).


pmoles taken up/mg dry weight
4000
3000
2000
1000
10
20
30
40
50
60
70
80
90
Time, min.

the control. These results were qualitatively the same as those obtained on assay at 37° after trypsin treatment of cells in phosphate buffer.

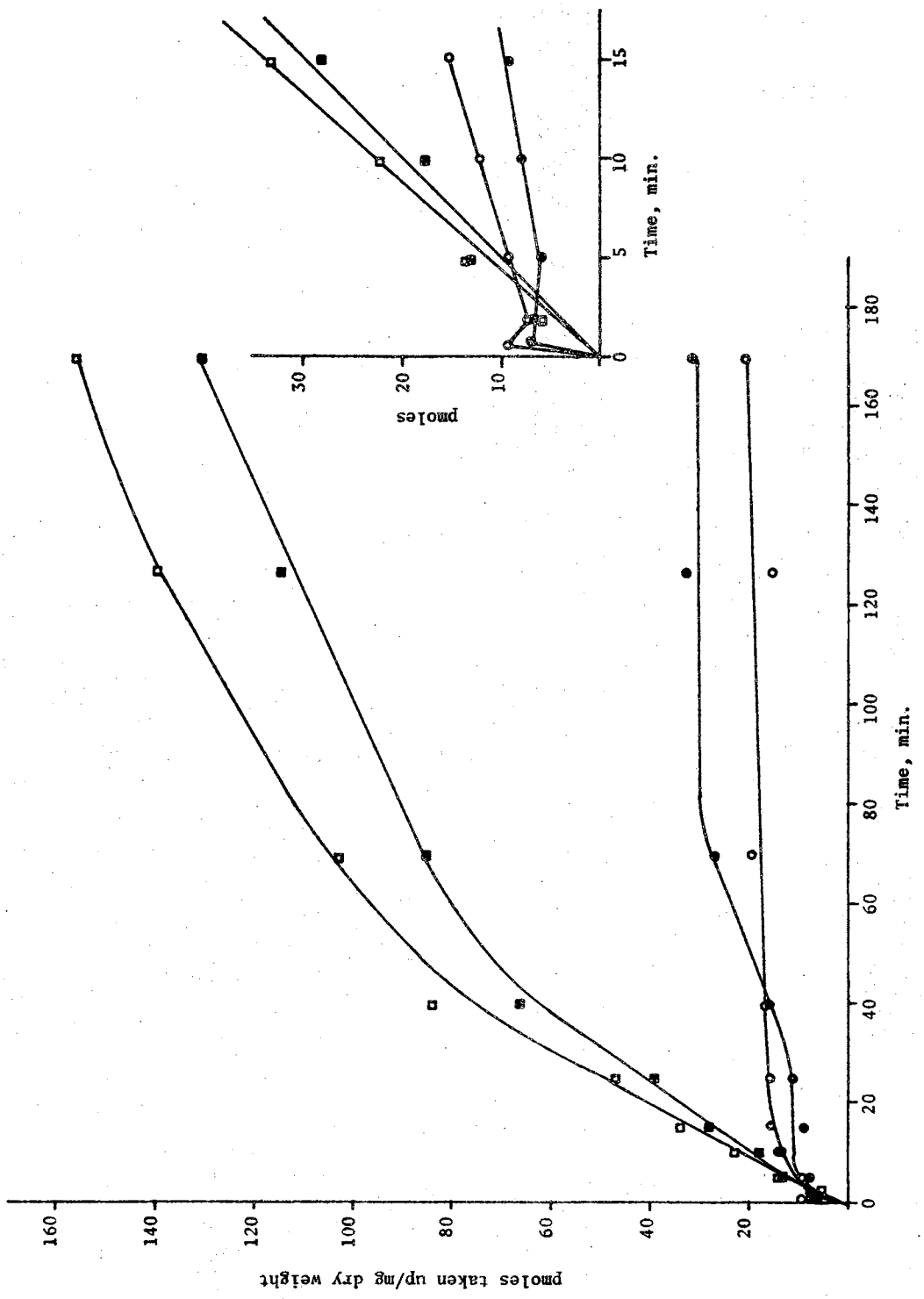
The trypsinization experiments just discussed were done on cells which exhibited high thiamine uptake capacities, accumulating over 1000 pmoles/mg dry cell weight. Trypsinization of cells with lower thiamine transport capacities produces a different effect. Figure 25 depicts the effect of trypsin at several concentrations under standard conditions on cells with less than 10% of the uptake capacity of those used in the previous experiments (these cells grew poorly in M-9). Instead of the reduced capacities seen in the earlier studies, a great enhancement of uptake capacity was effected by trypsinization of these cells. At 70 minutes the treated cells accumulated almost 4 times as much thiamine as the untreated cells. While stored on a slant, a cell type containing cryptic thiamine transport sites was selected. Trypsinization then altered the surface (or removed "protecting"protein), exposing the transport components to the medium.

This hypothesis was tested on intermediate capacity cells by varying trypsin concentration under the standard conditions. The results are shown in Table XX. Increasing trypsin concentrations effect increasing initial velocities and thiamine capacities up to a concentration of 10 µg/ml of trypsin. At higher concentrations initial velocity drops sharply and gradual decreases in capacity occur.

A time study using trypsin at 1 mg/ml gives a greater insight into the effect of trypsin action on this membrane activity. Figure 26 shows changes in initial velocity and accumulation at 60 minutes on treatment of low activity cells with trypsin for various lengths of time. A 5 minute treatment causes a drop to approximately half that of the control.

Figure 25. Effects of Trypsin Concentration on Uptake by Low Activity Cells

A late log phase M-9 culture of cells from an isolated colony on tryptone agar plate was harvested by Millipore filtration, washed with 10 mM Tris, pH 8.0, and suspended in the same buffer. Trypsinization was performed at a trypsin concentration of 1 mg/ml for 1 hr at 37°. Termination of reaction and uptake were done by the standard procedure. ● = Control (no trypsin), O = 0.1 µg/ml, ■ = 10 µg/ml, □ = 1.0 mg/ml trypsin.


pmoles taken up/mg dry weight
Time, min.
pmoles
Time, min.

Subsequent samples treated at longer times show a roughly linear increase in initial velocity. Treatments 45 minutes or longer gave velocities greater than those of the control. Accumulation at 1 hour and 2 hours followed the same pattern except that at the longest treatment times the activity reached a saturation.

TABLE XX

EFFECT OF VARYING TRYPSIN CONCENTRATION ON UPTAKE

Trypsin Concentration, µg/ml	$V_{5\ min.}$ pmoles/min / mg dry weight	Thiamine Accumulated at 73 Min. pmoles / mg dry weight
None	23.3	557
0.1	27.9	630
1.0	30.9	1024
10	41.8	1192
100	33.1	1093
1000	28.5	1004

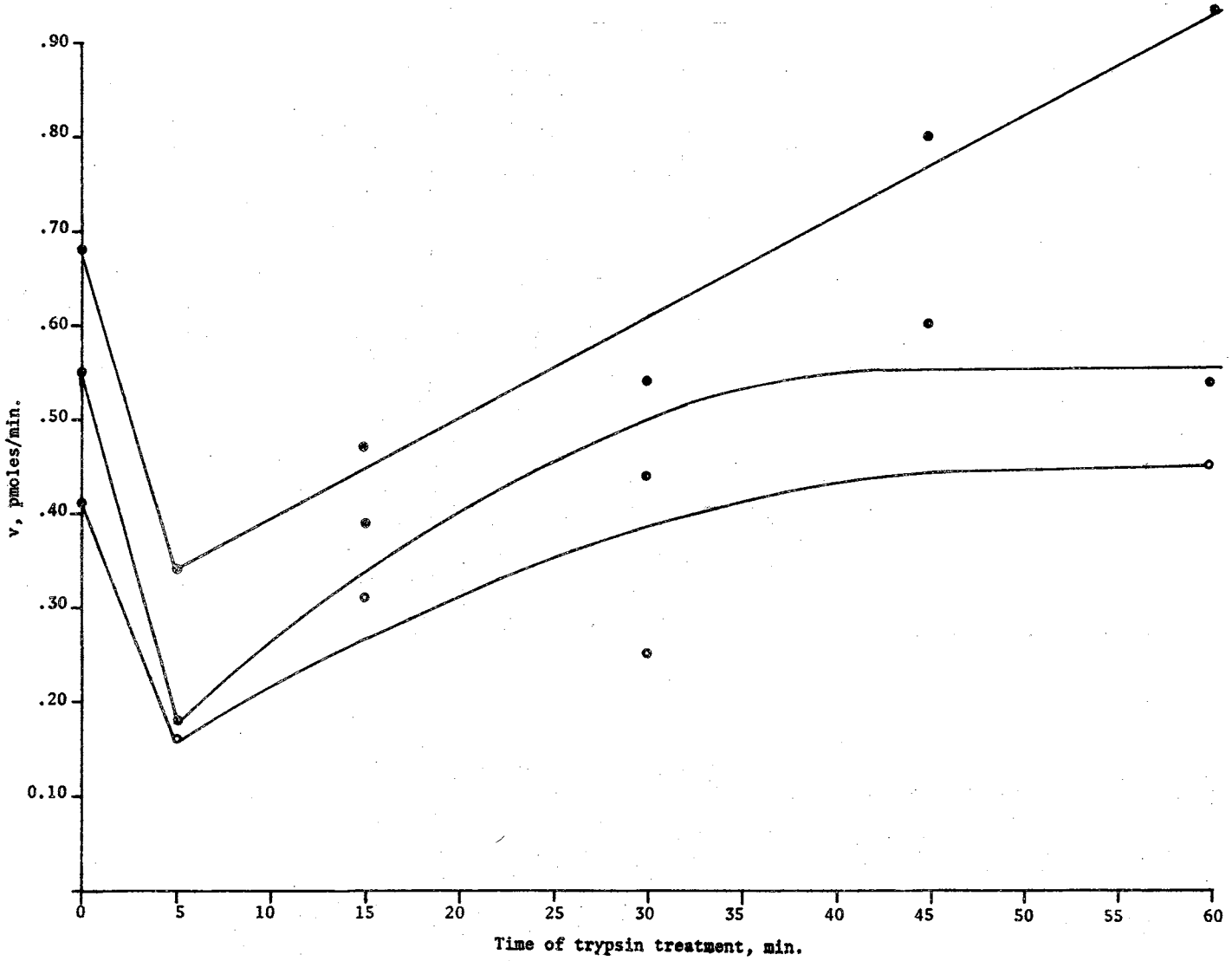
A log phase culture was harvested by Millipore filtration, washed with 10 mM Tris, pH 8.0, and suspended in the same buffer. Trypsinization was performed at 37° for 1 hr. Termination of reaction and uptake were done by the standard procedures.

These results show that the effects of trypsin treatment of cells on thiamine uptake are dependent upon 1) thiamine uptake capability of the cells, and 2) the conditions used in proteolysis. In cells having a high transport capacity mild trypsin digestion increases the initial

Figure 26. Effect of Time of Trypsin Treatment

Late log phase cells were harvested in the usual manner and suspended in 50 mM Tris, pH 8.0. Control samples were removed and placed in tubes containing trypsin (1 mg/ml) inactivated by 0.2 mM PMSF and filtered after two minutes. Trypsin was added to the reaction vessel to a concentration of 1 mg/ml. Aliquots of the reaction mixture incubated at 37° were taken at appropriate intervals and the reaction terminated by adding cells to tubes containing PMSF. These tubes were allowed to incubate for 2 min. before filtration and washing on Millipore filters.

After all samples had been collected, the cells on the filters were suspended in uptake medium and uptake assayed by the usual procedure. Aliquots were taken at 5, 18, 60 and 120 min. Initial velocities were obtained from the 5 and 18 min. samples.


.90
.80
.70
.60
.50
.40
.30
.20
0.10
v, pmoles/min.
0
5
10
15
20
25
30
35
40
45
50
55
60
Time of trypsin treatment, min.

velocity of uptake but depresses maximal accumulation. More stringent conditions, e.g., higher trypsin concentrations, higher temperature or longer treatment times, decrease both initial velocities and maximal accumulation.

Trypsinization of low activity cells produces the opposite effects. Even under the most stringent conditions of trypsin concentration, temperature and time of digestion accumulation was greatly enhanced by trypsin treatment. The time of trypsinization study (Figure 25) shows a decrease in initial velocity only at the shortest times of treatment.

Intermediate activity cells show behavior which indicates that there are sites at the surface which are accessible to trypsin and sites which are buried or "cryptic". Mild trypsin treatment conditions evoke a response similar to that of low activity cells, whereas stronger treatment elicits behavior similar to that seen with high activity cells.

These results give some insight into the surface localization of components involved in the different aspects of the complex thiamine transport process. Normal activity cells appear to have a component responsible for the slower accumulation exposed, whereas a component involved in more rapid accumulation is partially buried. This component is then exposed on trypsinization. Low activity cells apparently have few sites of either type available at the surface until trypsinization.

CHAPTER VI

DISCUSSION

Thiamine transport in E. coli Crookes is an active process; it is temperature-dependent (Figure 5 and Table XV) and requires exogenous energy source for maximal accumulation (Figures 8 and 19). It is not inducible by thiamine in the growth medium (Table XVI); the initial velocity of thiamine uptake and cellular accumulation are decreased in thiamine-grown cells. Growth of cells on adenine, which derepresses thiamine biosynthetic enzymes, increases the initial velocity of thiamine uptake but decreases cellular thiamine levels. These results agree with those of Kawasaki and Esaki (93) for E. coli K-12.

The results reported in this thesis on whole cells and isolated membrane vesicles demonstrate that thiamine uptake in this organism is a complex process involving two or more separate transport mechanisms, one for accumulation at low exogenous thiamine levels (< 0.10 μM) and one or more for higher concentrations. The time course of uptake in whole cells at low thiamine concentrations is hyperbolic, whereas at higher concentrations more complex curves are seen (Figure 17), indicating a faster and a slower process. The uptake curves at higher concentrations are similar to those obtained by Ames for histidine transport (7). With membrane vesicles there was an immediate saturation, followed after a variable time interval by linear accumulation (Figures 4 and 5). This immediate saturation is temperature-independent, occurring to the same

extent at 4° as at 37° (Figure 5). Double reciprocal plots on data obtained from initial velocity studies on whole cells (Figure 18) and on membrane vesicles (Figure 6) and from equilibrium data on vesicles (Figure 7) all show qualitatively the same effect on variation of thiamine concentration. A pronounced break in the curves occurs between 0.10 and 0.15 μM thiamine. In vesicles higher thiamine concentrations produce a substrate activation effect such that K_m's cannot be obtained.

Non-hyperbolic plots of accumulated thiamine versus thiamine concentration were obtained in equilibrium studies under optimal conditions with membrane vesicles (Figure 8). Under suboptimal conditions decreases in uptake occurred concomitant with a double saturation. A number of conditions produced this biphasic curve: omission of energy source or Mg^{2+} (Figures 8 and 10); substitution of Ca^{2+} for Mg^{2+} or addition of EDTA (Figure 10); addition of a number of inhibitors, including cyanide, fluoride, azide and thiamine pyrophosphate (Table II); or treatment with chaotropic agents such as detergents or phenethyl alcohol (Table VI). The curves obtained with membranes from whole cells treated with these inhibitors and chaotropic agents were qualitatively and quantitatively similar to those obtained routinely from shocked cell membranes. These results suggest that the activity remaining in whole cell membranes after these treatments is binding rather than accumulation; shocked cell membranes are probably fragments rather than vesicles and are incapable of accumulating thiamine. The diversity of the methods for suppression of the active accumulation process in whole cell membranes may suggest that this effect occurs via a number of different mechanisms. However one mechanism which could be common to all is uncoupling of transport components from interaction with the energy production mechanisms which

normally provide them with energy required for concentration against a gradient. Shocked cell membranes, rather than being fragments, could also be vesicles whose surface structures have been so altered on osmotic shocking that energy production mechanisms have become irreversibly uncoupled from the transport components which they energize.

Maximal accumulation in both whole cells and vesicles is dependent upon Mg^{2+} (Figures 10 and 21). In shocked cell membranes addition of Mg^{2+} decreases K_{d_1} 5-fold and K_{d_2} 1.5-fold, yet the maximal thiamine level at the first saturation is decreased 3-fold and at the second saturation almost 2-fold (Table VII). It is difficult to assign definitive roles for an ion as ubiquitous as Mg^{2+}. However, the apparently disparate Mg^{2+} effects can be integrated into a mechanism consistent with data on whole cells and both types of membranes. One can assume from the data that Mg^{2+} enhances accumulation by two mechanisms, 1) enhancement of binding to both components or sites and 2) facilitation of release of the bound thiamine into the interior of the cell or membrane vesicle. Thus in the whole cell and the whole cell membrane, which retain their barrier function, enhancement of binding and facilitation of release of thiamine both increase accumulation in the interior. On the other hand in shocked cells, even though Mg^{2+} enhances thiamine binding, it also aids in release; since these membranes apparently do not retain their barrier function, thiamine is liberated back into the medium.

Both whole cells and membrane vesicles show differential effects of pH on accumulation. Membrane vesicles at pH 6.6 show greater levels of accumulation at lower thiamine concentrations than at pH 7.8 or 9.0. There is little effect of these pH's at concentrations greater than 0.15 μM (Figure 11). Uptake studies on whole cells show increased

initial rates at pH 6.6 in Tris, but decreased maximal accumulation compared to that at pH 8.0 (Table XVII). These differential effects of pH on initial velocity and maximal accumulation are further evidence for a two-transport system capability of the cells for thiamine. The thiamine binding protein, with a pH optimum of 9.0, may be a component of the high pH system. An alternate explanation for the differential pH effects may involve an exit process which occurs preferentially at pH 6.6.

More information on the two postulated thiamine transport systems can be obtained by analysis of the results of energization and inhibition experiments. Glucose was the most effective energy source in stimulating transport in both whole cells and membrane vesicles; neither PEP nor lactate was as effective as glucose. This may indicate that 1) glucose is energizing more than one type of transport system, possibly both the D-lactate dehydrogenase system and the PEP-PTS, or 2) glucose is energizing a completely different system of transport instead of or in addition to these two systems. One possibility might be a transport system linked to glucose dehydrogenase. Such a system has been suggested for earlier work on E. coli W membrane vesicles in which glucose stimulation of transport was greater than that by other energy sources (94). Although glucose dehydrogenase is a partially soluble enzyme in this organism, Kaback suggests that the method of membrane preparation used in this work allowed retention of this enzyme, which then may have been involved in a transport system. This enzyme may be more tightly bound in E. coli Crookes such that it remains intact under the conditions of membrane preparation.

Results of inhibitor studies give greater insight into transport systems which may be functioning in thiamine transport in E. coli Crookes.

A summary of expected effects of various inhibitors on D-lactate dehydrogenase and PEP-PTS-linked transport and observed effects on thiamine transport is shown in Table XXI.

TABLE XXI

EFFECT OF INHIBITORS ON TRANSPORT SYSTEMS

Inhibitor	Expected Effect on Transport Via		Observed Effect on Thiamine Transport	
	PEP-PTS	D-LDH	Whole Cells	Membrane Vesicles
Cyanide	Decrease	Decrease (efflux)	Decrease	Decrease
Azide	Increase	Decrease (efflux)	Decreased v_i, Increased Accumulation	Decrease
2,4-DNP	Increase	Decrease (efflux)	Great Decrease	No Effect
Fluoride	Decrease	No Effect	Decrease	Decrease
Oxamate	No Effect	Decrease	Great Decrease	Increase

In whole cells cyanide decreases both the initial velocity of thiamine uptake and thiamine accumulation capacity. The inhibition of transport by cyanide could be a result of inhibition of a D-lactate dehydrogenase-coupled system or inhibition of the PEP-PTS or other type of transport system dependent upon electron transport. On the other hand the differential effects of azide on transport by the two well-characterized systems provides a more useful means of ascertaining the functionality of these systems in transport of thiamine. In whole cells

azide inhibits initial velocity, suggesting that the fast process occurs via a transport system such as the D-lactate dehydrogenase which is inhibited by oxidative phosphorylation inhibitors. The dramatic inhibitory effect of oxamate on thiamine uptake in whole cells (Table XII) substantiates the conclusion that a significant portion of the thiamine transport capability in this organism occurs via the D-lactate dehydrogenase.

In contrast to the inhibitory effect of azide on initial velocity of thiamine uptake in whole cells, this inhibitor enhances thiamine accumulation capacity. This observation suggests that the PEP-PTS may account for a portion of the uptake, since PEP production is increased on uncoupling of oxidative phosphorylation from electron transport. Since cyanide and the enolase inhibitor fluoride show approximately the same quantitative effects in whole cells, i.e., inhibition of initial velocity and of accumulation (Table XIV), the transport activity inhibited by these could be attributed to the PEP-PTS or another system energized at a point prior to PEP, such as a glucose dehydrogenase system. Fluoride can also inhibit glycolysis at hexose phosphate isomerase and can complex hemes to inhibit electron transport. If a glucose dehydrogenase system functioned in thiamine transport, fluoride should increase its activity by shutting off the competitive breakdown of glucose through the glycolytic pathway. On the other hand it would tend to inhibit this mechanism by inhibiting electron transport. It is difficult to conclude on the available information whether a portion of the thiamine uptake capability is linked to a glucose dehydrogenase system.

Fluoride can complex with Mg^{2+} and phosphate and thus is capable of inhibiting also any Mg^{2+}-requiring non-metabolic proteins which might be

involved in transport. This inhibitor may affect a component directly involved in transport, such as the transport component discussed earlier which was postulated to be Mg^{2+}-dependent with respect to its affinity for thiamine.

Inhibitor studies on isolated membrane vesicles necessitate different conclusions concerning the transport systems involved in thiamine uptake. In contrast to the dramatic decrease in accumulation seen in the presence of oxamate in whole cells, this specific D-lactate dehydrogenase inhibitor has no inhibitory effect on accumulation in membrane vesicles. These vesicles may have lost a component of the D-lactate dehydrogenase-coupled system which undoubtedly functions in thiamine accumulation in whole cells. The thiamine binding protein, which is very loosely attached to the cell and is probably lost on membrane preparation, may be a component of the D-lactate dehydrogenase-coupled system. Alternatively, structural alteration of the membrane surface during preparation of membrane vesicles, may lead to inaccessibility of D-LDH system thiamine-specific component(s).

The effect of azide on accumulation in vesicles is just the opposite of its effect in whole cells. This uncoupler, which may stimulate accumulation by the PEP-PTS in whole cells, dramatically inhibits accumulation in membrane vesicles. This fact indicates that another system inhibitable by uncouplers is functioning in the vesicles.

Trypsin treatment of isolated membrane vesicles and whole cells afforded some insight into the surface localization of components involved in thiamine transport. Pronase treatment has been used by Passow (95) to determine accessibility of glucose transport components to the surface of erythrocyte membranes. Treatment by trypsin is a more subtle

approach because of this enzyme's limited specificity. An extensive study of conditions of trypsin treatment in the present study allowed conclusions to be drawn concerning more subtle transport component interactions which may be important in regulating the amount of thiamine taken up by the cells.

Mild trypsinization of membrane vesicles decreases thiamine accumulation at thiamine concentrations less than 0.10 μM, while significantly increasing levels above that concentration. Treatment for longer periods of time gave no more change at the lower thiamine concentrations but decreased levels at the higher concentrations from the levels seen after treatment for the shorter time (Table III). These results indicate that a component or site for binding thiamine at lower concentrations is partially exposed to the surface such that it is inactivated by trypsin treatment. Concomitantly a second site which is partially buried within the membrane becomes exposed, either by removal of "protecting" protein or by conformational alteration, such that it is more accessible for interaction with thiamine. Further trypsinization then begins to degrade the newly exposed sites, and at longer times decreases accumulation below control levels.

The effect of trypsin treatment on thiamine uptake in whole cells depended upon the amount of that activity in the untreated cells and the conditions of trypsin treatment (Figures 23 through 26). Mild treatment of high activity cells increased initial velocity while depressing maximal accumulation; more stringent conditions of treatment decreased both initial velocity and maximal accumulation. Trypsinization of low activity cells under even the more rigorous conditions enhanced both initial velocity and maximal accumulation of thiamine. Trypsin treatment of

cells with intermediate thiamine uptake capability gave decreased uptake on short intervals of treatment; further trypsinization produced a linear increase in both initial velocity and maximal accumulation. These data indicate that fewer transport sites are available at the surface of the lower activity cells. These cryptic sites are then uncovered or otherwise exposed by trypsin treatment so that they are accessible for binding and transport of thiamine.

This approach can be used to great advantage in studying effects of energy sources and inhibitors on localization of carriers with respect to the surface of the membrane. Kepes (96) has suggested that energized carriers during transport are resistant to inactivation by NEM and FDNB, whereas these carriers, under normal energization, in the absence of ligand are inactivated by these reagents. His conclusions are tenuous, since these reagents do penetrate the membrane barrier. The use of trypsin treatment and treatment by other highly specific enzymes would give less equivocal answers to the questions concerning localization.

A vast number of studies over the past decade have centered on transport of nutrients across membrane barriers. Most of these have employed whole cells, but studies with isolated membrane vesicles which retain transport capability are increasing. These membranes are obviously not identical to the membrane of the intact cell, since both protein and lipid are lost during the process of preparation. Although these membranes have been characterized with respect to retention of their barrier function (52), and some gross comparisons have been made on uptake capacities and rates between whole cells and isolated membranes (46), to date no in-depth comparisons have been made to ascertain whether isolated membranes can be truly regarded as first derivatives of whole

cell membranes such that their use exclusively in studying transport phenomena is valid. Cell perturbations such as osmotic or cold shock will release factors which bind nutrients with great affinity and which have been implicated in their respective transport processes. Few successes in reconstitution of lost transport activity by adding back these purified components have been achieved. There may well be a structural reason for this failure, therefore negative reconstitution results should not a priori rule out the involvement of these proteins in transport. The present studies have indicated gross functional differences among whole cells and membranes isolated from intact cells and shocked cells. These differences could conceivably result from only minor structural alterations, especially if these alterations cause loss of components which are functional in transport.

There are very obvious physical differences between membranes isolated from normal and from shocked cells. Shocked cell membranes show a much greater adhesiveness than do normal cell membranes, and this property results in aggregation effects not seen in the latter type. Uptake in whole cell membranes is a linear function of membrane concentration, whereas shocked cell membranes show a complex non-linear concentration dependence. Functional comparisons between the two membrane types give further evidence of gross differences. Activities are much lower in shocked cell membranes and show no dependence upon exogenous energy source as do normal cell membranes. These membranes behave as fragments incapable of accumulation against a gradient rather than as semipermeable membrane vesicles.

Gross functional differences between normal cell membranes and whole cells also are evident. The diverse effects of 2,4-dinitrophenol, azide,

fluoride and oxamate on isolated membranes and whole cells suggest that isolated membranes have lost a functional portion of a transport system for thiamine which is coupled to the D-lactate dehydrogenase system. Thus in the whole cell fluoride, an inhibitor of enolase, does not diminish the thiamine transport capability to a great extent, whereas in isolated membranes thiamine accumulation is virtually annihilated in the presence of this inhibitor. Oxamate, on the other hand, shows no inhibitory effect in isolated vesicles, yet inhibits a great percentage of the activity in whole cells. It appears that during membrane preparation a component of the D-lactate dehydrogenase transport system found in the whole cell is lost. It is not likely that the D-lactate dehydrogenase itself is lost, since it is tightly bound to the membrane in E. coli (54). A more reasonable candidate would be a specific component involved in the transport of thiamine such as the thiamine binding protein. Binding proteins which may function in transport processes may be lost easily on preparation of membranes. The loss of the galactose binding protein on preparation of vesicles may account for the differences in transport of galactose observed in isolated vesicles and in whole cells (43,44). Structural alterations of the membrane surface in preparing vesicles may also cause altered transport properties. Such surface alterations may account for the wide-spread failure in reconstituting transport by addition of purified binding proteins to shocked cells. The studies presented here indicate that caution should be used in extrapolating information on transport obtained using isolated membrane vesicles to transport as it functions in the intact organism, unless parallel studies are performed on the whole cell.

CHAPTER VII

SUMMARY

Thiamine transport in Escherichia coli Crookes was studied in isolated membrane vesicles from normal cells, in membranes from shocked cells and in whole cells. Transport is an active process in vesicles from normal cells and from whole cells; membranes isolated from shocked cells were probably fragments, since there was no energy requirement and an aggregation effect resulting in nonlinear accumulation with membrane protein concentration was present.

Isolated membrane vesicles from normal cells show energy- and temperature-dependent uptake; glucose is the most effective energy source. D,L-Lactate gave only a 5% increase in levels accumulated over membranes in the absence of exogenous energy source. Accumulation was greatly decreased by azide, fluoride and cyanide; 2,4-dinitrophenol had little effect on maximum accumulation and oxamate, a specific D-lactate dehydrogenase inhibitor, stimulated accumulation by 20%. The competitive inhibitor thiamine pyrophosphate at low TPP/thiamine ratios (1:1) decreased accumulation of thiamine at thiamine concentrations less than 0.10 μM and at higher concentrations accumulation was affected very little. A 10:1 TPP/thiamine ratio effected a great decrease in accumulation at all thiamine concentrations.

Mg^{2+} is required for maximal thiamine accumulation; Ca^{2+} will not replace Mg^{2+} in promoting accumulation below 0.30 μM thiamine, and levels

are much less in the presence of Ca^{2+} than in membranes with no exogenous metal ion. At thiamine concentrations greater than 0.30 μM, however, accumulation begins to increase linearly until at 0.50 μM it approaches that in the presence of Mg^{2+}. EDTA at a concentration of 10 mM greatly decreases thiamine levels.

At pH 6.6 accumulation is optimal for thiamine concentrations less than 0.15 μM. Raising the pH to 7.8 or 9.0 moderately decreases accumulation at thiamine concentrations less than 0.10 μM, greatly decreases levels at 0.10 and 0.12 μM and above this concentration levels return to those at pH 6.6. Thus the lower portion of the thiamine concentration curve is pH-dependent, the higher portion pH-independent over the range studied. At lower and higher thiamine concentrations accumulation increases linearly with membrane protein concentration.

One noteworthy feature of the equilibrium curves is that they are non-hyperbolic. Under normal conditions one saturation is seen, while biphasic saturation appears under most suboptimal conditions. Factors which will cause this obvious biphasic curve are absence of energy source or Mg^{2+}, Ca^{2+} substitution for Mg^{2+}, presence of EDTA, fluoride, azide and TPP, elevation of pH and freeze-thawing. Addition of any energy source would result in a single saturation; cyanide did not produce the biphasic curve.

Trypsinization of membranes produces varying effects on thiamine accumulation. Under mild conditions lower thiamine concentration accumulation is decreased, with a concomitant increase in higher concentration levels. At longer incubation times there was no change in levels at the first saturation concentrations, but the second saturation levels were decreased from the shorter incubation time levels. Under more extreme

conditions thiamine levels were decreased at both saturations.

From these results it was concluded that a component or site of a system for accumulation of lower thiamine concentrations exists at the membrane surface in an arrangement such that it is available to thiamine and only moderately available to trypsin. A second component or site for accumulation of higher concentrations, which is normally partially buried within the membrane, is exposed by trypsin treatment. This effect may be on removal of covering protein or an alteration of conformation of protein to expose the second site. Further trypsinization then degrades this second exposed site.

Accumulation by shocked cell membranes was much less than in normal cell membranes and was not a linear function of membrane protein concentration. All conditions under which equilibrium studies were done gave a pronounced biphasic binding curve with thiamine concentration. Thiamine accumulation was energy-independent and was concluded to be simple binding, since the membranes appeared to have lost their permeability barrier.

Thiamine transport in whole cells is an energy- and temperature-dependent process whose kinetics are complex. At higher concentrations (> 0.1 μM) a somewhat biphasic uptake curve is seen, whereas at 0.10 μM Michaelis-Menten type kinetics occur. The system is Mg^{2+}-dependent for maximal accumulation. Azide decreases initial velocity but stimulates accumulation at later times. Cyanide, fluoride and DNP decrease both initial rates and accumulation; the most profound decrease in these is seen in the presence of oxamate.

Glucose is the most efficient energy source. Neither D,L-lactate nor PEP is as effective in stimulating rates or accumulation. Thiamine

transport in whole cells appears to occur by both the D-lactate dehydrogenase system and the PEP-PTS or a system energized at a step prior to the enolase reaction. This energy linkage may occur through glucose dehydrogenase.

Effects of trypsinization of whole cells on thiamine uptake are dependent on uptake capability of the cells and conditions of trypsin treatment. Mild conditions produce enhanced initial velocity and decreased maximal uptake in cells with normal activity; more rigorous conditions decrease both parameters. Trypsinization of cells with low thiamine uptake capacity causes stimulation of both initial velocity and maximal capacity in a treatment time-dependent manner. These results are consistent with the hypothesis that normal activity cells have at their membrane surfaces a component for slower accumulation and a cryptic component for the faster system. Low activity cells, on the other hand, contain two components which are cryptic; activity in these cells occurs only on exposure of these by trypsinization.

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